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**AUTOANTIBODIES TO CENTROSOMES ARE DIAGNOSTIC FOR HUMAN
SCLERODERMA AND CAN BE INDUCED BY EXPERIMENTAL
MYCOPLASMA INFECTION IN MICE**

A Dissertation Presented

By

IRINA CATRINEL GAVANESCU

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences, Worcester
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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IMMUNOLOGY AND VIROLOGY

**AUTOANTIBODIES TO CENTROSOMES ARE DIAGNOSTIC FOR HUMAN
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ABSTRACT

The overall objective of this thesis work was to develop new insights into the etiology of scleroderma, a human systemic autoimmune disease, by analyzing the autoantibodies to centrosome antigens that develop during the disease. Centrosomes are perinuclear organelles that form microtubule arrays, including mitotic spindles that ensure the faithful segregation of chromosomes during mitosis.

These studies used a novel methodology to determine the prevalence of anti-centrosome autoantibodies in patients with scleroderma. Recombinant centrosome antigens were used to determine the antigenic specificity of anti-centrosome antibody subsets by immunoblotting. Centrosome marker antibodies were used in indirect immunofluorescence assays to distinguish centrosomes within the polymorphic staining pattern frequently given by scleroderma sera. We found that 43% of patients are autoreactive to centrosomes, a prevalence higher than has been reported for any other scleroderma autoantigen. Half of the centrosome-positive patients also had autoantibodies against other antigens used in scleroderma diagnosis. However, in the remaining half of these patients, anti-centrosome antibodies represented the sole class of autoantibodies that was detectable. Anti-centrosome antibodies were detected in only a small percentage of normal individuals and patients with other connective tissue diseases.

These data suggest that anti-centrosome autoantibodies may represent a new diagnostic tool in scleroderma. Upon examination of anti-centrosome autoantibody development in an animal model, it appeared that this autoantibody specificity may develop in mice as a consequence of an infection.

An infectious agent was isolated by plaque-formation from carrier mice. Further characterization of the infectious agent was undertaken to obtain information on its physical, morphological and cytopathological properties. The infectious agent was identified by sequence and unique antigenic properties to be homologous to the pig pathogen *Mycoplasma hyorhinis*. When reintroduced into naive mice, the murine mycoplasma triggered anti-centrosome autoantibody development. While anti-centrosome autoantibodies of IgM isotype are part of the repertoire of naive unimmunized mice, mycoplasma infection specifically triggered the development of anti-centrosome IgG. Moreover, centrosome autoreactivity was prevented by antibiotic treatment. The autoantibody response evolved to recruit additional specificities, having IgM isotypes, reactive to endoplasmic reticulum-associated autoantigens.

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LIST OF ABBREVIATIONS

ACR, American College of Rheumatology

ACA, Anti-centromere

ANA, Anti-nuclear antibodies

BSA, Bovine serum albumin

CP140, Centrosome protein of 140 kDa, centriolin

CD, Cluster of differentiation

C, Complement

CR, Complement receptor

°C, Degrees Celsius

DNA, Deoxyribonucleic acid

DAPI, 4,6-diamidino-2-phenylindole

DMEM , Dulbecco's minimal essential media

EMEM, Earl's minimal essential media

FITC, Fluorescein isothiocyanate

Gadd45a, Growth arrest and DNA damage-inducible gene

HRP, Horseradish peroxidase

IF, Immunofluorescence

Ig, Immunoglobulin

IL, Interleukin

NCBI, National Center for Biotechnology Information

PBS, Phosphate-buffered saline

PCR, Polymerase chain reaction

PKC δ , Protein kinase C δ

Rag, Recombinase activating gene

RT, Room temperature

SAP, Serum amyloid P

snRNP, Small nuclear ribonucleoprotein

SDS, Sodium dodecyl sulfate

SLE, Systemic lupus erythematosus

SSc, Systemic sclerosis,

TH, T helper

TBS, Tris buffered saline

TCR, T cell receptor

CHAPTER I

INTRODUCTION

A. Specific Aims

The overall goal of this dissertation was to further our understanding of scleroderma, a human connective tissue disease. Our approach was based on the principle that elucidating the mechanism that leads to the development of a key scleroderma feature like unique autoantibodies will provide insights into the as yet unknown etiology of the disease. The goal of this work was to analyze a specific subset of autoantibodies that recognize proteins of the centrosome, and to examine the loss of tolerance to centrosome autoantigens in human scleroderma patients and in a murine model demonstrating similar autoantibody development. When autoantibodies to centrosomes were found to develop in mice exposed to an infectious agent, the overall objectives of the study were expanded to include the isolation, identification and characterization of that infectious agent and to demonstrate that naive mice develop characteristic centrosome autoantibodies when infected with that agent.

The specific aims of this study were:

1. To characterize autoantibodies to centrosomes that develop in patients with scleroderma; this required the following:
 - to design sensitive and specific assays to detect human anti-centrosome autoantibodies

- to determine anti-centrosome autoantibody prevalence in scleroderma
- to determine if anti-centrosome autoantibodies were unique to scleroderma patients or appeared in patients with other connective tissue diseases or in normal individuals
- to define the diagnostic and prognostic significance of anti-centrosome autoantibodies detected in scleroderma

2. To develop an animal model for the study of anti-centrosome autoantibodies. This aspect of my work included:

- the isolation of a putative infectious agent from autoantibody-positive mice
- the identification of the isolated infectious agent by genome sequence and antigenic properties
- the characterization of the physical, morphological and cytopathological properties of the infectious agent in vitro
- the determination of the ability of the infectious agent to induce anti-centrosome autoantibodies in vivo

3. To establish the conditions necessary to induce an infection-dependent autoantibody response in mice including the determination of:

- the route of infection
- the kinetics of the response
- the isotypes recruited during the response
- the autoantibody specificities that developed upon infection

The following introductory material will place the objectives of this study in the context of our current knowledge of the mechanisms of autoantibody development, the role of autoantibodies in human systemic autoimmune diseases, and the induction of autoantibodies as a consequence of infection.

B. Mechanisms of Autoantibody Development

The development of high-titer autoantibodies is a consistent feature of many human autoimmune diseases. However, autoantibodies can be also found in healthy individuals, suggesting important differences between disease-related autoantibodies and autoantibodies with roles in normal physiology.

Autoantibodies in Normal Physiology. Low titered autoreactive immunoglobulins, called “natural autoantibodies” and produced by CD5-expressing B cells, are readily detected in the sera of healthy individuals (Stall et al., 1996). Natural antibodies are mainly of the IgM isotype and use variable region genes that are not somatically hypermutated, whereas disease-associated autoantibodies have switched to multiple isotypes and have undergone somatic hypermutation (Bona and Rothfield, 1994; Feldmann et al., 1996; Kotzin, 1996; Stall et al., 1996). Natural autoantibodies are not pathogenic and are thought to fulfill a housekeeping function, e.g. clearing modified self-antigens that result from cellular apoptosis or oxidized lipoprotein (Shaw et al., 2000). B cells producing natural autoantibodies are likely positively selected on self-antigen, and

they frequently produce antibodies that cross-react with bacterial antigens (Hayakawa et al., 1999; Shaw et al., 2000). This reactivity to non-self antigen postulates an additional role for natural antibody in innate immunity. Indeed, a natural antibody reactive to both self and non-self antigens has been shown to provide optimal protection from infection with virulent bacteria (Briles et al., 1982; Shaw et al., 2000).

Natural autoantibody secretion can be induced by stimulation of receptors other than the B cell antigen receptor. Bacterial lipopolysaccharide signalling through Toll-like receptors induces autoantibody secretion by CD5-positive B cells (Hayakawa et al., 1999; Kawai et al., 1999). Similarly, CpG dinucleotide-containing bacterial nucleic acid can also induce polyclonal B cell activation (Hemmi et al., 2000). It is likely, therefore, that natural autoantibody production can be induced by a number of non-self components.

As mentioned previously, pathogenic autoantibodies found in autoimmune disease are class switched and somatically mutated. Paradoxically, these processes normally occur in germinal centers, and natural autoreactive B cells and conventional autoreactive B cells are normally excluded from entering germinal centers in the presence of cognate self-antigen (Cyster et al., 1994; Stall et al., 1996). However, a very recent paper showed that in an autoimmune mouse strain autoreactive B cells undergo active somatic hypermutation outside of germinal centers (William et al., 2002). This suggests that autoantibodies result during autoimmune disease upon abnormal recruitment of

autoreactive B cells into germinal centers or due to ectopic isotype switching and somatic hypermutation, outside of germinal centers.

It is possible that disease-related autoantibodies result from the pathologic somatic hypermutation of natural autoreactive B cells. Such an event is implied by the striking similarity of specificities recognized by the natural autoantibody repertoire to those found in the high titer autoantibodies developed by systemic autoimmune disease patients (Hansen et al., 2000). Alternatively, autoantibody production during autoimmune disease may result from pathologic somatic hypermutation and/or survival of conventional autoreactive B lymphocytes. Autoantibody responses of conventional B lymphocytes will be outlined below.

Autoantibodies Can Develop Due to B Cell Epitope Mimicry. During immune responses to foreign antigens, conventional B lymphocytes routinely generate immunoglobulins that cross-react with self and non-self epitopes. In one study, lupus-like antibodies to double stranded DNA (dsDNA) of IgG isotype resulted upon immunization with phosphorylcholine, a pneumococcal cell wall component (Ray et al., 1996). Moreover, a monoclonal antibody with dual specificity for dsDNA and phosphorylcholine is able to recognize a third epitope, a peptide from a phage display library (Gaynor et al., 1997). Immunization with this peptide results in the development of anti-dsDNA antibodies (Putterman and Diamond, 1998). During infection, somatically mutated autoreactive antibodies are produced by a significant fraction of the B cells

recruited into the immune response (Ray et al., 1996). These autoantibodies can trigger pathology similar to that found in systemic lupus erythematosus (SLE) (Putterman and Diamond, 1998). However, autoreactive B cells activated during immune responses to foreign antigen are normally short-lived and destined to undergo apoptosis (Ray et al., 1996). Induction of a milieu supporting the survival of autoreactive lymphocytes activated as a consequence of infection could account for durable autoantibody development and autoimmune pathogenesis. Under these circumstances, conventional B cells can generate autoantibodies as a consequence of infection, due to the homology between self and non-self epitopes.

Autoantibody Responses Can Be Initiated by Immunogenic, Although Previously Cryptic B Cell Epitopes and Can Diversify by Epitope Spreading. Autoimmune disease patients develop autoantibodies to distinct epitopes found on interacting proteins of multimolecular complexes (Craft and Fatenejad, 1997). The presence of multiple antigenic targets in a multimolecular complex indicates that the entire complex is taken up and individual components are presented on the same antigen-presenting cell. Several studies also reported that intact, native macromolecular complexes are more frequently targeted by patient autoantibodies than the individual antigen components of which they are comprised, and many patient autoantibodies recognize conformational determinants (Craft and Fatenejad, 1997) (Kasturi et al., 1995).

Although the autoantigens of multimolecular complexes targeted in a systemic autoimmune disease may have very distinct subcellular locations, they frequently cluster together within vesicular surface structures produced in apoptosing cells (Casciola-Rosen et al., 1994). During virus-induced apoptosis, viral antigens co-cluster with autoantigens of systemic autoimmune diseases, providing a mechanism by which infection may render autoantigens immunogenic (Rosen et al., 1995). This proximity, combined with apoptosis-specific cleavage of autoantigens by proteases or reactive oxygen species, may explain the production of new immunogenic, previously cryptic epitopes (Casciola-Rosen et al., 1995).

Autoantibodies to complex antigens first seem to react with one or a few epitopes and evolve in predictable ways to include more epitopes, defining a phenomenon named epitope spreading (Shlomchik et al., 2001). An example of this process is provided by native small nuclear ribonucleoprotein (snRNP) particles, which are normally not immunogenic. However, after priming mice with either of two self-peptides encoded by snRNP antigens, challenge with the native murine snRNP produced antibodies that immunoprecipitated snRNP (Bockenstedt et al., 1995). This suggests that loss of tolerance to self can be initiated in the absence of foreign antigen by the abnormal processing and presentation of one initiating epitope; subsequently, epitope spreading within the same molecule (intramolecularly) or within neighboring molecules (intermolecularly) can account for the amplification of the autoantibody response

(Bockenstedt et al., 1995; Topfer et al., 1995). Epitope spreading might be induced by activated autoreactive T cells (Craft and Fatenejad, 1997).

Normal B Cell Tolerance May Be Broken by the Presence of Activated Autoreactive T Cells. A role for autoreactive T cells in the development of human systemic autoimmune disease is suggested by the presence of activated T cells at sites of chronic inflammation (White, 1996). Also, pathogenic autoantibodies are somatically mutated and class-switched, both normally T cell dependent processes (Craft and Fatenejad, 1997). Moreover, T cells might directly cause tissue damage. Lupus-prone mice have interstitial nephritis consisting of T cell infiltrates similar to human SLE patients (Shlomchik et al., 2001). In mice prone to autoimmune disease, peripheral T cells had a significantly lower threshold for activation than those from normal mice. As expected, genetic knockouts of T or B cells showed a requirement for both cell subsets for the development of SLE.

A direct demonstration of the role of T cells in systemic autoimmune pathogenesis was provided when it was shown that mice expressing a transgenic autoreactive T cell receptor (TCR) developed rheumatoid arthritis (Kouskoff et al., 1996). T cells in this system help autoreactive B cells, which, in turn, secrete pathogenic autoantibodies. Autoreactive B cells are normally quiescent in the absence of the transgenic T cells, and mice do not spontaneously develop rheumatoid arthritis. The fact that disease is initiated in this system by a homogeneous T cell population bearing the same transgenic TCR indicates that a critical number of autoreactive T cells are mandatory for pathogenesis.

Once initiated, pathology is mediated entirely by autoreactive immunoglobulin, in conjunction with complement (Korganow et al., 1999).

In other studies T cells have been shown to influence autoantibody-mediated pathogenesis by acting on natural autoreactive B cells, also called B1 cells. Some T helper 2 (TH2) cytokines (IL-5 and IL-10, but not IL-4) induce B1 cell differentiation to autoantibody-producing cells (Nisitani et al., 1995). In another study $\gamma\delta$ T cells induce differentiation of B1 cells to autoantibody-producing cells by non-cognate interactions (Watanabe et al., 2000). The development of autoreactive B and T cells with an activated phenotype is increasingly attributed to genetic factors.

Multiple Genetic Factors Facilitate Autoantibody Production. Autoantibody development has been observed in mice with homozygous mutations of the following: complement components C1, C2 or C4; receptors for complement CR2; cell death mediators Fas, Fas ligand and perforin; or mediators of negative signalling like the growth arrest and DNA damage-inducible gene (Gadd45a), protein kinase C δ (PKC δ) and transcription factor T-bet (Chen et al., 2000; Ishigatsubo et al., 1988; Miyamoto et al., 2002; Peng et al., 1998; Peng et al., 2002; Prodeus et al., 1998; Salvador et al., 2002; Taylor et al., 2000). Mutations that impede the clearance of apoptotic cells also trigger autoantibody development. Mice deficient in the serum amyloid P (SAP) component, which binds degraded chromatin fragments of apoptotic and necrotic cells, develop anti-nuclear antibodies (Bickerstaff et al., 1999). Similarly, mice lacking a functional “Mer”

membrane tyrosine kinase develop anti-nuclear autoantibodies and a lupus-like syndrome as a consequence of impaired phagocytosis and clearance of apoptotic cells (Cohen et al., 2002; Scott et al., 2001). Components of the classical complement pathway also participate in the clearance of apoptotic cell debris, and their absence, consistently detected in autoimmune disease patients, may contribute to the development of autoantibodies (Taylor et al., 2000). Autoreactive antibodies also develop in mice transgenic for B lymphocyte activators like CD19, which regulate the signaling threshold of autoreactive B cells (Sato et al., 2000). Autoantibody development has an incomplete penetrance in any of these systems, pointing to the role of additional, environmental factors for its initiation.

Pathogenic Role of Autoantibodies. Autoantibodies have characteristics that reflect on the mechanism of disease development and can be pathogenic themselves. It is therefore important to define the autoantibody properties that are necessary and sufficient to confer a pathogenic potential. In the MRL/lpr mice, pathogenic autoantibodies result from oligoclonal, antigen-receptor-mediated expansion of B lymphocytes that became somatically mutated (Shlomchik et al., 1990). However, somatic mutation is not obligatory, as unmutated antibodies were found to be both capable of high-affinity binding to self-antigens and pathogenic (Shefner et al., 1991). The affinity of autoantibodies is not a predictor of their pathogenic potential (Hansen et al., 2000). Moreover, low affinity autoantibodies can trigger cell lysis by binding to repetitive structures on target cell surfaces (Fossati-Jimack et al., 1999). The effector functions

mediated by the predominant isotype found in autoimmune disease may be informative of the mechanism by which pathogenesis is mediated. For example, different isotypes have different abilities to bind complement or Fc receptors and provoke tissue damage (Azeredo da Silveira et al., 2002).

Pathogenic autoantibodies are, by definition, capable of inducing specific autoimmune disorders when transferred into normal hosts. A growing body of evidence has described the specificities of several pathogenic autoantibodies. For example, autoantibodies to dsDNA induce immune complex glomerulonephritis (Raz et al., 1989; Tsao et al., 1990). Autoantibodies to glucose-6-phosphate isomerase, a cytoplasmic glycolytic enzyme, can provoke rheumatoid arthritis when injected into normal mice (Matsumoto et al., 1999). Anti-erythrocyte and anti-thrombocyte antibodies can trigger autoimmune hemolytic anemia and autoimmune thrombocytopenic purpura, respectively (Harrington et al., 1990; Horwitz, 1979a; Horwitz, 1979b). In pemphigus vulgaris, anti-desmoglein antibodies recognize an extracellular matrix component, desmoglein (Ding et al., 1999). It is noteworthy that, while autoantibody specificity is of key importance in defining pathogenic potential, the intra- or extracellular localization of the autoantigen is irrelevant. It is not clear, however, how antibodies to intracellular antigens will cause disease. It has been suggested that such antibodies cross-react with self antigens on cell surfaces (Lunardi et al., 2000).

The specificity of autoantibodies is critical for their pathogenicity in paraneoplastic

syndromes. Paraneoplastic syndromes are disorders that accompany malignancies and are mediated by pathogenic autoantibodies developed to tumor-specific neoantigens. Pathology results from the cross-reaction of autoantibodies with normal tissues. Antibody-induced paraneoplastic syndromes are numerous and polymorphic, suggesting that autoantibody-mediated pathology is not fully appreciated (Lang and Vincent, 1996). It appears that the specificity of an autoantibody is the major feature that defines its pathogenic potential. Human autoimmune diseases are characterized by a multitude of autoantibody specificities, as discussed below.

C. Autoantibodies in Systemic Autoimmune Diseases

Patients with systemic autoimmune diseases develop autoantibodies of many different specificities (Sontheimer et al., 1992; Tan et al., 1988). Autoantibodies precede or parallel disease onset and have been linked to events that initiate chronic inflammatory pathology (Sontheimer et al., 1992; Tan et al., 1988). Each autoimmune disease has autoantibodies with a distinct specificity profile (Hiepe et al., 2000).

A large number of studies have used patient sera to identify the molecular structure of autoantigens, in hopes of determining unique as well as common, structural or functional features that would explain their antigenicity (Galperin et al., 1996). The unifying feature of autoantigens recognized in systemic autoimmunity is that they are phylogenetically conserved, ubiquitous intracellular proteins, often assembling in larger macromolecular

complexes (Hemmerich and von Mikecz, 2000). Although some self-epitopes bear homology to viral or bacterial sequences, the significance of these examples of “molecular mimicry” for systemic autoimmune pathogenesis has not been clarified; not all sera from patients with antibodies to the autoantigen in question recognize the cross-reactive epitope (Okano, 1996).

Because autoantibody induction closely parallels disease onset, autoantibody detection reliably orients diagnosis (Hiepe et al., 2000). The prevalence and disease-specificity of autoantigens have been extensively described (Galperin et al., 1996; Hiepe et al., 2000). None of the reported autoantibody specificities is present in all patients with one systemic autoimmune disease. While no one autoantibody specificity is exclusively associated with a single disease, certain specificities are more characteristic of one autoimmune disease versus another (Galperin et al., 1996). Within one autoimmune disease, distinct autoantibody specificities correlate well with certain disease features or clinical subsets of the disease and have a predictive value on disease severity (Tan et al., 1988).

Systemic autoimmune diseases have autoantibodies of many specificities. These occur early during disease development and may have diagnostic significance. Similarly, scleroderma autoantibodies show a restricted specificity for intracellular organelles, are found early in disease, and may be of significant diagnostic or prognostic value.

Autoantibodies in Scleroderma. Scleroderma (systemic sclerosis, SSc) is a chronic disease of unknown etiology involving the vascular system, connective tissue and immune system. Three types of lesions are characteristic: vascular insufficiency, excessive scar-like deposition of extracellular matrix proteins, and chronic inflammation; these lesions develop in the skin, the musculoskeletal system and internal organs like the lung, the gastrointestinal tract, the heart and the kidney (Jimenez et al., 1996; LeRoy, 1996; White, 1996).

Vascular lesions are the initiating event in scleroderma pathogenesis. Overt pathology is preceded by an intermittent vasospasm called Raynaud's phenomenon, followed by endothelial cell injury (LeRoy, 1996). Evidence is accumulating to support the hypothesis that the endothelium is the target of an immune attack (Lunardi et al., 2000; Sgonc et al., 1996). The fully developed vascular pathology consists of capillary destruction and a hypertrophy of the vascular intima in arteries and arterioles. Progressive collagen deposition is found in scleroderma and likely results from overproduction of cytokines IL-4 or TGF- β during chronic inflammation (Jimenez et al., 1996). The presence of chronic inflammation in conjunction with the development of autoantibodies to intracellular antigens (bearing the generic name of anti-nuclear autoantibodies, ANA) and the lack of a single target organ assigns scleroderma to the spectrum of systemic autoimmune diseases. Further support for scleroderma being an autoimmune disease is provided by patients who develop overlapping syndromes, that cumulate clinical features of scleroderma and clinical features of another systemic autoimmune disease like

systemic lupus erythematosus, Sjogren syndrome, dermatomyositis etc. In addition, scleroderma has a close clinical and histopathological resemblance with graft-versus-host disease, a condition with an obvious immune pathogenesis (White, 1996).

Scleroderma Diagnosis. Scleroderma epidemiology is similar to that of other systemic autoimmune diseases, in that the typical scleroderma patient is three times more likely to be a woman between the ages of 30 and 50 years at disease onset. Scleroderma affects all races and has a worldwide distribution. The disease has an ethnic bias, with blacks and Choctaw Native Americans being more frequently affected (Gilliland, 1998). The disease prevalence has been reported to be between 19 and 75 per hundred thousand individuals (Gilliland, 1998).

Familial histories of scleroderma patients show that the disease recurs in families 11 times more often than expected (Tan and Arnett, 2000). The occurrence within the same family of cases with SSc and cases with other autoimmune diseases such as SLE has been frequently reported (Tan and Arnett, 2000). Scleroderma does not have a high degree of concordance among monozygotic twins, indicating that in addition to genetic factors, environmental factors may be also important in disease development (Tan and Arnett, 2000). Interestingly, the aggregation of scleroderma autoantibodies within the families of autoimmune disease patients is significantly higher, with first-degree relatives as well as spouses of patients being more frequently affected (de Juan et al., 1994). This finding, too, suggests a role for environmental factors in scleroderma pathogenesis.

Scleroderma diagnosis is established both clinically and histopathologically. The histopathologic hallmark of scleroderma is the presence of a proliferative reaction of the vascular intima (Jimenez et al., 1996). Mononuclear inflammatory infiltrates characteristic of early skin lesions are similar to infiltrates encountered in graft versus host disease. They appear in the immediate perivascular area and consist primarily of T cells and to a lower extent of B cells (White, 1996). The additional detection of characteristic autoantibody specificities also greatly aids scleroderma diagnosis (Gilliland, 1998). Scleroderma is clinically heterogeneous, but has been classified in two major clinical forms: diffuse and limited, which differ in the extent and severity of organ involvement (van den Hoogen and de Jong, 1995). The diffuse disease has widespread organ involvement and rapid progression, while the limited form of the disease is confined to the skin of extremities, and internal organs are involved late.

Scleroderma Autoantibodies. Characteristic autoantibodies of scleroderma are specific for DNA topoisomerase I, centromere proteins, and RNA polymerases I, II, or III. None of these autoantibody specificities are found exclusively in scleroderma, but are detected, although rarely, in other autoimmune diseases as well (Galperin et al., 1996). Nor is any one of these specificities detected in all scleroderma patients, but only in subsets comprising approximately 25% of patients (Bona and Rothfield, 1994). Although scleroderma patients respond to numerous other autoantigens, any one of these other specificities is at lower prevalence when compared to the above listed autoantigens (Bona

and Rothfield, 1994; White, 1996). Some autoantibody specificities correlate with the diffuse and limited forms of the disease and have therefore not only diagnostic, but also prognostic significance. Anti-centromere antibodies are found primarily in limited scleroderma, while anti-topoisomerase antibodies correlate with diffuse disease. Furthermore, some nucleolar antigens have been proposed as markers for visceral involvement (Okano, 1996). Autoantibodies in overlap syndromes between scleroderma and other systemic autoimmune diseases cumulate autoantibody specificities characteristic for each disease (Gilliland, 1998).

There is limited evidence for autoantibodies being directly pathogenic in scleroderma. Autoantibodies can induce endothelial cell apoptosis in vitro and in a scleroderma disease model (Lunardi et al., 2000; Sgonc et al., 1996). Given the precedent of autoantibody-mediated pathology in lupus and rheumatoid arthritis, scleroderma autoantibodies may have the potential to cause or exacerbate disease. Alternatively, scleroderma autoantibodies may be epiphenomena and occur due to the presence of activated autoreactive T cells. In either case, the identity of the autoantigen that initiates loss of tolerance may be revealed by the autoantibody specificity present at disease onset and detectable in all or the majority of patients.

Animal Models for Scleroderma. The most faithful scleroderma animal model is provided by University of California at Davis (UCD) chicken lines (L) 200 and 206, which develop a hereditary systemic connective tissue disease that closely resembles

scleroderma (Sgonc et al., 1996; Van de Water et al., 1995). Like human scleroderma, the avian disease is characterized by vascular occlusion, perivascular lymphocytic infiltration of the skin and viscera and antinuclear antibodies. UCD L200 chickens also display an abnormal thymic architecture (Van de Water et al., 1995). Although they develop a vigorous autoantibody response, UCD L200 chicken do not develop the autoantibody specificities considered to be characteristic of human scleroderma (Van de Water et al., 1995). In addition, the avian disease differs from scleroderma by being rapidly progressive and having a high lethality (Van de Water et al., 1995).

Mice bearing the autosomal tight skin (tsk) mutation on chromosome 2 provide an alternative animal model for scleroderma. These mice share with the human disease a thickening of the skin due to increased collagen deposition and the development of scleroderma-like autoantibodies. In contrast to the human disease however, autoantibodies in these mice occur at 8 months of age, well after the onset of the tight skin phenotype (Bona and Rothfield, 1994; Van de Water et al., 1995). No suitable animal model is currently available to study the initiation of scleroderma-like autoantibody responses.

Autoantibodies to Centrosomes in Scleroderma. One intracellular target of autoantibodies in scleroderma is the centrosome, a peri-nuclear organelle that duplicates during mitosis ensuring the faithful segregation of chromosomes (Doxsey, 2001). Autoantibodies to the centrosome, to the centrosomal core, the centriole, or to the mitotic spindle have been reported in scleroderma at low prevalences 4-6% (Tuffanelli et al.,

1983). Interestingly, the few scleroderma patients reported to have centrosome autoreactivity displayed only anti-centrosome autoantibodies, suggesting that autoreactivity to centrosomes may develop early, and may actually initiate scleroderma pathogenesis (Conrad and Mehlhorn, 2000; Doxsey et al., 1994; Moteki et al., 1991; Osborn et al., 1982).

We developed two novel immunoassays using recombinant centrosome proteins and centrosome-specific antibodies to demonstrate that a significant proportion of scleroderma patients exhibit centrosome reactivity. We investigated the prevalence and disease-specificity of anti-centrosome autoantibodies by screening a large cohort of scleroderma patients and comparing them with normal individuals and patients with other connective tissue diseases. We found that centrosome autoantibodies develop in 43% of scleroderma patients, a prevalence significantly higher than has been reported for any other scleroderma autoantigen (Gavanescu et al., 1999). Early data on centrosome reactivity was likely an underestimate due to assay insensitivity and to the very small patient cohort examined (Tuffanelli et al., 1983). Interestingly, early data from a few case reports also correlate anti-centrosome autoantibodies with infection (Auer-Grumbach and Achleitner, 1994; Cimolai et al., 1994; Gentric et al., 1991; Huidbuchel et al., 1991; Lind et al., 1988; Moteki et al., 1991).

D. Infection and Autoimmunity Are Characterized by Autoantibodies

A link between infection, autoantibody production and systemic autoimmune disease has been explored since the 1950's (Walton, 1968).

Infection and Autoimmune Disease. The best example of infection-induced autoimmunity is the development of human rheumatic disease syndromes, predominantly arthritis, as a consequence of infection. Although such arthritis occurs in patients in conjunction with clinical, serological or microbiological evidence of an antecedent infection, no pathogen can be isolated from the affected joint (Hughes and Keat, 1994). Moreover, this "sterile" arthritis develops in multiple animal models as a direct consequence of infection with bacteria, most notably mycoplasma (Cole et al., 1975; Cole et al., 1976; Harwick et al., 1973). Self-limiting autoimmune syndromes frequently accompany human infections. Cold agglutinins (cryoglobulins) induced characteristically by mycoplasma and hepatitis C virus trigger autoimmune hemolytic anemia and vasculitis (Gerber et al., 1997; Lamprecht et al., 1999). Exacerbations of systemic autoimmune disease subsequent to patient infection have also long been noted (Herrmann et al., 2000). While no unequivocal serological or microbiological correlation between a pathogen and a human systemic autoimmune disease has been established to date, individual disease features, especially autoantibody development, have often been linked to infection.

Infection and Autoantibodies. Infectious agents are likely to elicit autoantibody production by multiple mechanisms. Bacterially or virally encoded superantigen could drive the activation and expansion of autoreactive B or T lymphocytes (Schiffenbauer et

al., 1998; Silverman, 1998; Wucherpfennig, 2001). Presentation of self-antigen may be modified during infection: infected cells undergoing apoptotic death are strong dendritic cell activators, and these may present self antigens in an immunogenic mode; necrotic cell death will only result in dendritic cells delivering tolerogenic stimuli (Fonteneau et al., 2002; Gallucci and Matzinger, 2001). A direct demonstration of these mechanisms in autoantibody development is still awaited.

Molecular mimicry is one mechanism that can account for autoantibody development during bacterial infection. Cross-reactive autoantibodies develop during Chlamydia infection due to homology between myocardial and Chlamydia peptides (Bachmaier et al., 1999). Disease, i.e. autoimmune myocarditis, results in this system upon immunization with peptides homologous for either the myocardial or bacterial antigens, while bacterial nucleic acid functions as an adjuvant (Bachmaier et al., 1999). A direct pathogenic role of these cross-reactive autoantibodies has not been tested in this system.

A possible pathogenic role has been examined for cross-reactive autoantibodies capable of binding both intracellular autoantigens and viral proteins. Such antibodies have been reported to impair the function of their target cells when added to cultures (Levin et al., 2002; Lunardi et al., 2000). It is unknown whether these antibodies would be pathogenic in vivo. Autoantibodies are likely to develop by multiple mechanisms during infection and may induce pathology.

Infection-induced systemic autoimmune disease autoantibodies. Numerous infectious agents have been correlated in humans with connective tissue disease autoantibodies

(Labarca et al., 1997; Mengarelli et al., 2000; Newkirk, 2002; Rider et al., 1997; Toyoda et al., 1997; Toyoda et al., 1999). However, only lupus-like autoantibodies are reproduced by animal models upon infection with various pathogens, most notably viruses. For example, lupus-like ANA are induced in mice by infection with murine cytomegalovirus (MCMV) and herpesvirus 68 (HV68), likely due to the ability of both herpesviruses to trigger polyclonal B cell activation (Bartholomaeus et al., 1988; Olding et al., 1976; Price et al., 1993; Sangster et al., 2000). It has been also suggested that rabbits infected with polyoma virus produce lupus-like autoantibodies to DNA (Flaegstad et al., 1988). With the exception of lupus-like autoantibodies, no connective tissue disease autoantibodies could be induced by experimental infection.

Autoantibodies to Centrosomes in Infection. Similar to other connective tissue disease autoantibodies, autoantibodies to the centrosome have been also correlated with human infections with EBV, HIV and mycoplasma (Auer-Grumbach and Achleitner, 1994; Cimolai et al., 1994; Gentric et al., 1991; Huidbuchel et al., 1991; Lind et al., 1988; Moteki et al., 1991). We have shown that autoantibodies to centrosome proteins can be induced through experimental exposure of mice to a mycoplasma. With time, the centrosome-specific reactivity expands to include autoreactivity to endoplasmic-reticulum autoantigens. These findings show that mycoplasmas induce complex autoantibodies in mice and suggest they may be cofactors in the pathogenesis of human autoimmune diseases.

CHAPTER II

CENTROSOME PROTEINS: A MAJOR CLASS OF AUTOANTIGENS IN SCLERODERMA

A. Abstract

Autoantibodies to intracellular antigens are a hallmark of autoimmune diseases, although their role in disease pathogenesis is unclear. Centrosomes are organelles involved in the organization of the mitotic spindle, and they are targets of autoantibodies in systemic sclerosis (SSc). We used recombinant centrosome autoantigens, centrosome-specific antibodies, and immunoassays to demonstrate that a significant proportion of SSc patients exhibited centrosome reactivity. Two centrosome proteins cloned in our laboratory were used to screen 129 SSc sera by Western blotting. The same sera were screened by immunofluorescence using centrosome-specific antibodies to distinguish centrosomes from the nuclear speckles commonly stained by SSc sera. Using these criteria, 42.6% of SSc patients were autoreactive to centrosomes, a larger percentage than reacted with all known SSc autoantigens. Most centrosome-positive sera reacted with both centrosome proteins, and half were negative for other routinely assayed SSc autoantibodies. By these criteria, we have identified a novel class of SSc autoreactivity. Only a small percentage of normal individuals and patients with other connective tissue diseases had centrosome reactivity. These results demonstrate that centrosome autoantibodies are a major component of autoreactivity in SSc and thus have potential in disease diagnosis.

Centrosome autoantigens may be useful in studying the development of autoantibodies and chronic inflammation in SSc and perhaps other autoimmune diseases.

B. Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by microvascular lesions, lymphocytic infiltrates and fibrosis of the skin and visceral organs (LeRoy, 1996; Mitchell et al., 1997; White, 1996). The majority of patients produce autoantibodies to proteins of intracellular organelles. While many of these autoantibodies are shared among other autoimmune diseases, some are known to be specific for SSc and thus serve as useful immunological markers in disease diagnosis (Galperin et al., 1996). The intracellular targets of SSc autoantibodies are found mainly in the nucleus (Okano, 1996). Among these are antibodies to DNA topoisomerase I and to centromere proteins on kinetochores of chromosomes. While both classes of autoantibodies are good indicators of disease, only anti-topoisomerase autoantibodies are associated exclusively with SSc and not found in other autoimmune diseases (Okano, 1996; Vazquez-Abad and Rothfield, 1995). Another organelle that is a target of autoantibodies in SSc is the centrosome (Tuffanelli et al., 1983).

Centrosomes are intracellular organelles that nucleate microtubules and organize the bipolar mitotic spindle during cell division, although little is known of the molecular components involved in these functions (Dictenberg et al., 1998; Doxsey et al., 1994;

Kellogg et al., 1994). To identify centrosome components, we previously utilized anti-centrosome autoantibodies from patients with SSc to screen cDNA expression libraries (Doxsey et al., 1994). In this way, we identified two centrosome proteins that we named pericentrin and CP140 (centrosome protein of 140 kDa) (Doxsey et al., 1994). Using recombinant proteins from these cloned centrosome antigens and antibodies specific for these molecules, we investigated the prevalence of centrosome autoreactivity by immunofluorescence (IF) and Western blotting.

Previous reports indicated that the prevalence of centrosome-specific autoantibodies in SSc was quite low (4-6%) compared to the prevalence of antinuclear antibodies (50-90%)(Tuffanelli et al., 1983). We believe that the reported centrosome reactivity was underestimated due to assay insensitivity and the small patient cohort examined. In this study, we re-examine the prevalence of anti-centrosome antibodies in scleroderma using two novel assays for centrosome autoantigen detection. The first involves the use of two distinct recombinant centrosome proteins for probing patient sera by the Western blot technique. The second is an IF assay that employs two unique modifications: the use of a centrosome-specific antibody to identify unequivocally centrosome reactivity of autoimmune sera in double-label IF reactions and a detergent prepermeabilization step that removes soluble cytoplasmic staining that often obscures the centrosome signal. As a consequence of these methodological optimizations, we show that autoreactivity to the centrosome is more prevalent in SSc than reactivities to all other individual SSc autoantigens.

C. Results

Analysis of Centrosome Reactivity by Immunofluorescence. The prevalence of centrosome autoreactivity was examined in 129 SSc patients by immunofluorescence on human cultured cells (HepG2) using an antibody to pericentrin as a marker for the centrosome (Doxsey et al., 1994). This antibody gave the characteristic centrosome pattern of one or two small fluorescent dots adjacent to or superimposed on the nucleus (Figs. 1A and D). A proportion of the SSc sera gave this same pattern and did not appreciably stain the nucleus or other cellular structures (Fig. 1B, A and B superimposed in C). Other SSc sera gave a complex pattern of multiple dots in and around the nucleus. In these sera, centrosome reactivity was scored as positive when the signal from patient sera (Fig. 1E) was coincident with that of the pericentrin label (Fig. 1D), giving a yellow color when the signals were superimposed (Fig. 1F). Using these criteria, 41.1% of sera from SSc patients had anti-centrosome autoantibodies.

Analysis of Centrosome Reactivity by Immunoblotting. An independent biochemical assay was used to analyze centrosome reactivity in the same cohort of SSc patients. This approach also enabled us to determine the molecular specificity of the reactivity identified by immunofluorescence. In this assay, recombinant pericentrin and CP140 were probed with SSc sera by Western blotting (Fig. 2A and B). Signals generated by the autoimmune sera used to identify these proteins in the original screen are shown in lanes

2 and 3 and they serve as positive controls for the test sera (Doxsey et al., 1994). Normal human sera are shown in lanes 1, 14 and 24 and serve as negative controls in this assay. An example of a test serum that did not react with either of the two centrosome proteins is shown in lane 19. Examples of positive test sera with variable levels of reactivity are shown in lanes 7-13, 21 and 23. The results of the Western blot assay demonstrate that autoreactivity to CP140 and pericentrin was 26% and 27% respectively (Fig. 3, columns A and B). The cumulative reactivity against both centrosome proteins was 30.2% (Fig. 3, column C). For comparison, the results from the morphological assay are shown in Fig. 3, column d. The cumulative reactivity of both techniques was 42.6% (Fig. 3, column E). Nearly all of the sera positive by the biochemical assay were also positive by immunofluorescence (not shown).

Comparison of Centrosome Autoreactivity with Other SSc Autoantigens. We next determined whether the centrosome autoreactive sera also had reactivity to other autoantigens. Forty-nine percent of these patients had additional reactivity to one or more of the following autoantigens: topoisomerase I, centromeres, double-stranded DNA, small nuclear ribonucleoproteins, Sm, La, and Ro (Galperin et al., 1996; Okano, 1996; Vazquez-Abad and Rothfield, 1995; Vazquez-Abad et al., 1994). Interestingly, 51% of patients with centrosome reactivity did not react with any of the other routinely assayed autoantigens. This group of patients with autoreactivity to centrosome proteins establishes a previously unidentified category of autoreactivity in SSc that could have diagnostic potential.

Centrosome Autoreactivity, Clinical Forms of the Disease, and Clinical Disease

Features. Of the patients autoreactive to centrosome proteins, we found no significant difference between patients with different clinical forms of the disease (Calcinosis, Raynaud's phenomenon, Esophageal dysmotility, Sclerodactily, Telangiectasias, CREST, limited or proximal scleroderma; $\chi^2 = 0.24928$, $0.882 > P > 0.005$). We also found no statistically significant difference among patients with centrosome reactivity that had lung involvement ($\chi^2 = 0.05$, $0.82 > P > 0.005$), heart involvement ($\chi^2 = 0.11$, $0.73 > P > 0.005$), or involvement of the gastrointestinal tract ($\chi^2 = 0.5$, $0.48 > P > 0.005$); this cohort of patients was not uniformly characterized for renal involvement. No differences were noted when using data from immunofluorescence or immunoblotting or both combined.

Disease specificity of centrosome autoreactivity. To determine whether centrosome reactivity was specific for SSc, we screened sera from 73 normal individuals and 71 patients with other autoimmune diseases (Fig. 4). Of the 73 normal individuals investigated, only 2.7% were positive for anti-centrosome autoantibodies, a value significantly lower than the prevalence of centrosome autoreactivity in SSc patients ($Z = 4.6598$, $P < 0.00001$) and not significantly different from the 2% prevalence of ANA previously reported in the normal population ($Z = 0.4516$, $P > 0.3264$). Of the sera from 71 patients with other autoimmune diseases (see Chapter II), 11.1% had anti-centrosome autoantibodies. These included patients with SLE, rheumatoid arthritis and Sjogren's

syndrome. This result demonstrates that centrosome autoreactivity, while prevalent in SSc, is also found in some other autoimmune diseases, albeit at significantly lower frequencies ($Z=2.793$, $P<0.01$).

D. Discussion

High Prevalence of Centrosome Autoantibodies in SSc. A major finding of this study is that centrosome autoreactivity in SSc is higher than autoreactivity to all other known SSc autoantigens reported in the literature. This conclusion is based on the analysis of 129 patients with SSc, 71 patients with other autoimmune diseases, and 73 healthy individuals. The proportion of centrosome-reactive sera in this analysis (30-43%) was significantly higher than reported previously (4-6%) (Tuffanelli et al., 1983). This is likely due to three technical improvements: the development of a biochemical assay for centrosome autoantigens, the use of centrosome-specific antibodies, and a preextraction method for the IF assay to distinguish unambiguously centrosomes from nuclear speckles commonly stained by SSc sera.

The high level of centrosome autoantibodies detected in SSc sera is likely an underestimate since the truncated recombinant centrosome proteins used in the biochemical analysis were probably missing additional immunoreactive epitopes. Furthermore, additional centrosome autoantigens are known to exist since they were identified together with pericentrin and CP140 in the original screen (S. Doxsey, unpublished observations), but they have not yet been characterized or tested for

recognition by SSc sera (Doxsey et al., 1994). Judging by the imperfect overlap of immune responses to pericentrin and CP140 (Fig. 3), the untested centrosome proteins will likely increase the total centrosome reactivity found in SSc above the levels reported here.

Centrosome Autoantibodies as a New Diagnostic Tool for Scleroderma. A second major finding presented in this study is the exclusive occurrence of centrosome autoantibodies in half the SSc patients with centrosome autoreactivity. This observation demonstrates that centrosome autoantibodies define a novel subset of patients that lack reactivity to the autoantigens that are commonly used today to aid in clinical diagnosis (ACA and anti-topoisomerase antibodies). It will be interesting to determine how centrosome autoreactivity compares with reactivity to RNA polymerases, for which assays are not routinely employed in clinical diagnosis and which were not screened in this study (Okano, 1996).

The calculated sensitivity of the diagnostic test for centrosome autoantigens in SSc (0.30) is higher than that for ACA and anti-topoisomerase antibodies (0.26 and 0.25, respectively) (Okano, 1996; Vazquez-Abad and Rothfield, 1995).

Although encountered in other connective tissue diseases, the high disease specificity of centrosome autoreactivity (0.9726) and its high positive predictive value for SSc (0.95) indicate that this assay will provide an effective new diagnostic tool for scleroderma. When used together with assays for ACA and anti-topoisomerase, tests for anti-centrosome reactivity could add significant sensitivity to disease diagnosis.

Possible Mechanism for Generating Multimolecular Autoreactivity to Centrosomes.

The loss of tolerance to intracellular proteins in systemic autoimmune diseases such as scleroderma is not understood, although several mechanisms have been proposed. Among them is the hypothesis that autoreactivity to multiple protein components of an intracellular organelle may be the result of a loss of tolerance to an initiating self-epitope (Bockenstedt et al., 1995; James et al., 1995; Topfer et al., 1995). This epitope could be targeted by the immune system by virtue of sequence homology to a non-self antigen (e.g. a virus), a mechanism called molecular mimicry. Alternatively, this epitope could be cryptic and not exposed when self-proteins are presented to T and B cells in a tolerogenic mode (Bockenstedt et al., 1995). Cryptic epitopes could be exposed as a consequence of pathologic processes, such as vascular spasm in scleroderma. Ischemia, reperfusion, and the release of oxygen radicals that follow vascular spasm could cleave intracellular proteins and reveal cryptic epitopes (Casciola-Rosen et al., 1997).

Regardless of the mechanism, initial loss of tolerance to one epitope could be followed by sequential loss of tolerance to multiple epitopes of the same protein, a mechanism called intramolecular epitope spreading (Topfer et al., 1995). Results from this investigation and another study show that autoantibodies to multiple centrosome components are present together in the same sera (Figs. 2 and 3), consistent with intermolecular spreading (Mack et al., 1998). More work will be required to identify self-epitopes that initiate the autoimmune response to centrosome proteins during chronic inflammation in SSc and other autoimmune diseases. In addition, the mechanism by

which exclusive reactivity to one class of autoantigens is accomplished (ex. Centrosomes and not centromeres; Figs. 1A-C) is also an important remaining question.

Figure 1. Immunofluorescence images of cells co-labeled with pericentrin and SSc sera. (A) Pericentrin staining of HepG2 cells using a secondary fluorescein anti-rabbit IgG (green). (B) Autoimmune sera on the same cell using Cy3 anti-human IgG (orange). (C) An overlay of both channels showing coincidence of centrosome and autoantibody staining (yellow), together with DNA staining to visualize nucleus (blue). (D) Pericentrin staining as in A. (E) Autoimmune sera on the same cell showing numerous “nuclear speckles” present in most SSc sera (F). Overlay of both channels reveals centrosome reactivity (yellow).

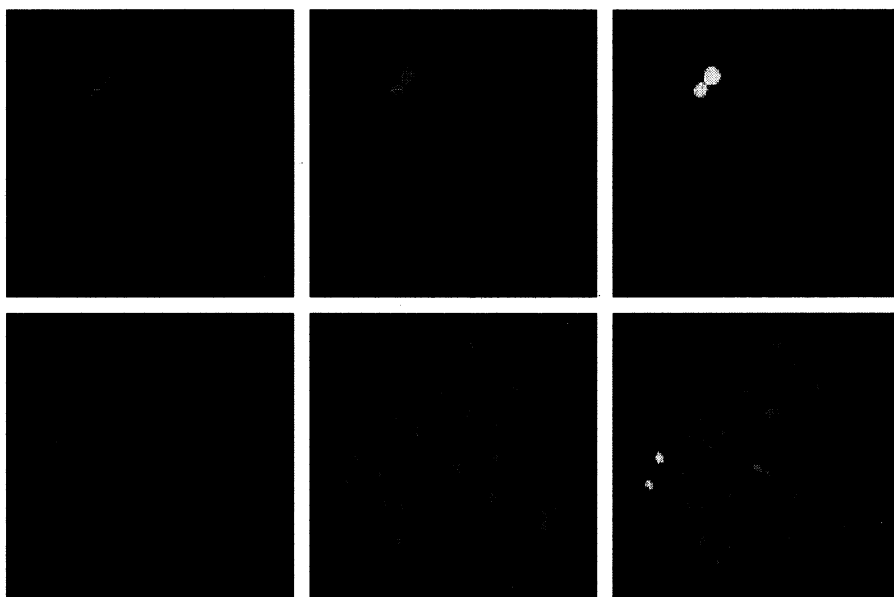


Figure 2. Immunoblots of recombinant centrosome proteins using SSc sera.

Recombinant pericentrin (A) and CP140 (B) were partially purified, separated on acrylamide gels, and transferred to Immobilon membranes. They were blotted with autoimmune sera or sera from normal individuals (controls). HRP-conjugated secondary antibodies raised against human IgG were used to detect primary autoantibodies.

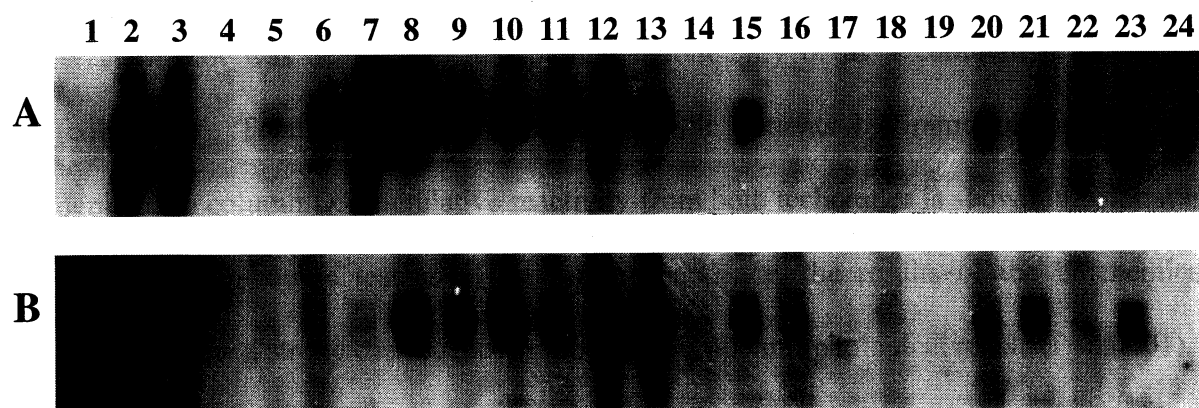


Figure 3. Immunoreactivity to CP140 and pericentrin in SSc. The percentage of patients positive for reactivity to CP140 (column A) or pericentrin (column B) by Western blot was determined and plotted individually or as a cumulative percentage (column C). Total centrosome reactivity of SSc patients by immunofluorescence is depicted in column D. Cumulative reactivity from both techniques is shown in column E. Using the chi-square test as a test for independence, the results of the biochemical analysis show a strong dependence compared with the morphological results($P<0.0001$).

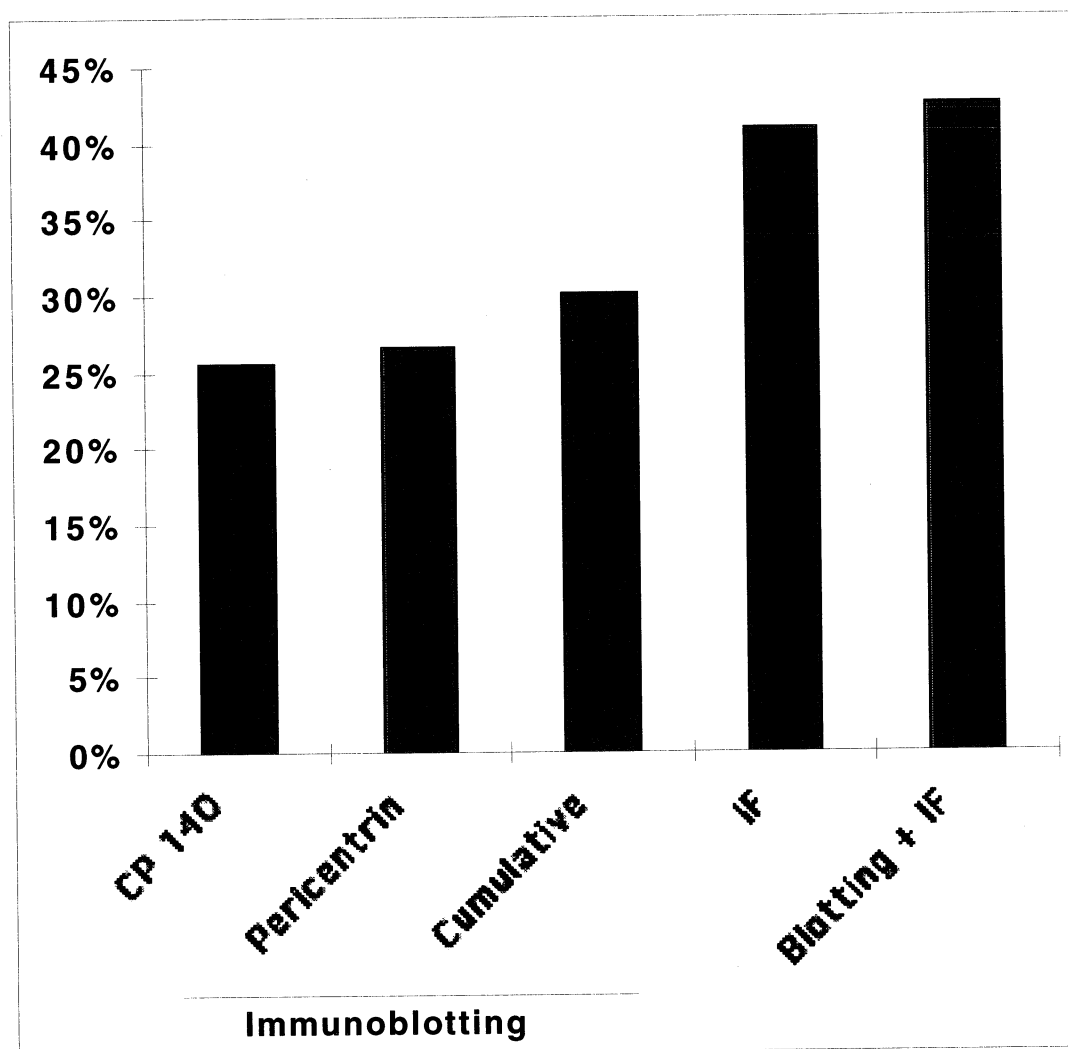
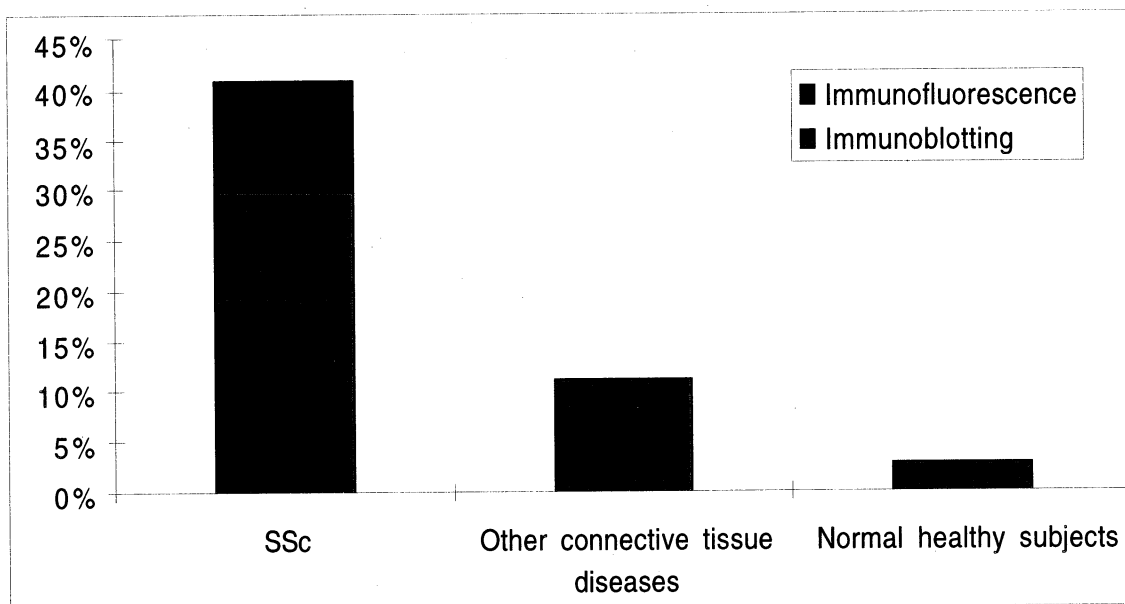


Figure 4. Disease specificity of centrosome autoreactivity. Centrosome autoantibodies were found in 30-43% of SSc sera. In other connective tissue diseases, centrosome reactivity was 11%. Centrosome autoreactivity was present in only 2.7 % of the normal population, a value similar to that described for other autoantigens.



CHAPTER III

SCLERODERMA-LIKE FEATURES IN MICE: MYCOPLASMA INDUCES CENTROSOME AUTOREACTIVITY THAT SPREADS TO OTHER ORGANELLES

A. Abstract

Development of autoantibodies to intracellular molecules is a universal feature of autoimmune diseases and parallels the onset of chronic inflammatory pathology. We recently demonstrated that the major target of autoantibodies in scleroderma are centrosomes, organelles that orchestrate mitotic spindle assembly in dividing cells. Here we demonstrate that this novel class of autoantibodies can be induced in mice following exposure to mycoplasma. Unlike other experimentally induced autoantibodies, autoreactivity was initially specific for centrosomes, later spread to include other intracellular targets and in the process class switched from IgM to IgG isotypes. Antibiotic treatment of mice prevented centrosome autoantibody development. Our findings demonstrate that mycoplasma induces complex autoantibody responses in mice, providing support for the idea that these microorganisms contribute to autoantibody development and pathogenesis in human autoimmune diseases and suggesting that they may represent novel therapeutic targets.

B. Introduction

Development of autoantibodies to a wide variety of organelles and proteins is an early feature of most human autoimmune diseases (Hiepe et al., 2000; Sontheimer et al., 1992; Tan et al., 1988). However, little is known about the events that initiate autoantibody development or the mechanisms that sustain their production. Even less is known about the mechanisms that control targeting of specific antigens and organelles during humoral autoimmune responses.

Infectious agents have been implicated in the development of autoantibodies in human diseases (Bach et al., 1998; Walton, 1968). Infections exacerbate pathological symptoms of many autoimmune diseases and can induce chronic autoimmune syndromes in both humans and animal models of human disease (Cole et al., 1975; Cole et al., 1976; Harwick et al., 1973; Hiepe et al., 2000; Hughes and Keat, 1994). Autoantibodies characteristic of some systemic autoimmune diseases correlate with infection (Newkirk, 2002; Rekvig et al., 1997; Rose, 2001) and in some cases can be induced by experimental infection with viruses, bacteria or parasites (Flaegstad et al., 1988; Olding et al., 1976; Sangster et al., 2000).

Several mechanisms have been proposed to link infection and autoantibody development. In the molecular mimicry model, autoantibodies are induced upon infection by peptide

antigens on pathogens that share homology to self-peptides (Bachmaier et al., 1999; Ray et al., 1996). In a broader sense, structural similarity of pathogen and self components can account for the stimulation of common receptor(s) to activate autoreactive B lymphocytes. For example, the same Toll-like receptors can be triggered by structurally related pathogen and self components and initiate autoantibody production through B cell receptor-independent pathways (Vinuesa and Goodnow, 2002). The autoantibody response can be specific rather than polyclonal if low doses of stimulatory molecules or immune complexes co-engage the appropriate Toll-like and antigen receptors (Coutinho and Moller, 1974; Leadbetter et al., 2002). These pathways have been implicated in triggering autoantibody production in response to non-self and self molecules including proteins, polysaccharides, lipopolysaccharides, lipoprotein and nucleic acid (Hacker et al., 2000; Kawai et al., 1999; Li et al., 2001; Lien et al., 1999).

There is currently little evidence to support a role for infectious agents in the pathogenesis or autoantibody development in scleroderma, a human systemic autoimmune disease characterized by chronic inflammation of the skin and visceral organs (Gilliland, 1998). Self epitopes recognized by autoantibodies in scleroderma patient sera share sequence homology to epitopes on retrovirus and herpesvirus antigens (Jimenez et al., 1995; Lunardi et al., 2000). However, these cross-reactive viral epitopes have not been shown to act as dominant targets of immune responses during viral infection nor have they been shown to initiate an immune response to self antigens during scleroderma pathogenesis. One limitation to this type of analysis is the lack of an

experimental system to test whether infectious agents can directly initiate autoantibody responses to scleroderma autoantigens.

Centrosomes are inconspicuous organelles approximately 1 μ m in diameter that form microtubule arrays in interphase cells for controlling cell shape and polarity, and they organize microtubule spindles in mitotic cells to ensure proper chromosome segregation (Doxsey, 2001). We recently discovered that centrosomes are the major autoantibody target in scleroderma (Fauci, 2001-2002; Gavanescu et al., 1999). The high prevalence of centrosome autoreactivity was somewhat surprising given the low level of centrosome autoreactivity previously reported in autoimmune diseases (Kellogg et al., 1994). Unambiguous identification of centrosomes was achieved by using more sensitive and specific centrosome assays and demonstrated that these tiny organelles have important diagnostic potential in scleroderma.

In this study, we show that mycoplasma infection is associated with centrosome autoantibodies and that isolated mycoplasma can directly trigger centrosome-specific autoantibodies when introduced into naive mice. Centrosome autoreactivity expands with time to include other intracellular organelles and is prevented by treatment with antibiotics. These results provide the first evidence for a scleroderma-like autoantibody response that is initiated by the loss of tolerance to centrosome autoantigens and occurs as a consequence of infection.

C. Results

We recently showed that centrosomes are a previously unappreciated autoantibody target in human scleroderma (Gavanescu et al., 1999). Centrosome autoantibodies are more prevalent than all other autoantibodies in this disease making them an important diagnostic indicator and suggesting that they may play a role in disease pathogenesis (Fauci, 2001-2002). To understand more about centrosome autoantibody development, we examined the phenomenon in a murine experimental system. Using morphological and biochemical assays to detect centrosomes and centrosome autoantigens (Doxsey et al., 1994; Gavanescu et al., 1999), we observed sporadic centrosome autoreactivity in several mouse colonies. Up to 35% of animals in some colonies had centrosome autoantibodies while other colonies had undetectable levels of centrosome autoreactivity (Table I).

The sporadic occurrence of centrosome autoreactivity in mice was consistent with a model in which autoantibodies developed by transmission of an infectious agent. To test this hypothesis, we asked whether autoantibody reactivity could be initiated through contact with infected animals. We unexpectedly found that naive mice housed with sero-positive but not sero-negative mice rapidly developed a specific autoantibody response to centrosomes and centrosome antigens (Fig.5).

Characteristics of an Infectious Agent that Induces Anti-Centrosome Autoantibodies. To isolate the putative infectious agent responsible for centrosome autoantibody development, we prepared suspension homogenates from mouse spleens and used them to inoculate cultured mammalian cells. Plaques formed in cell monolayers inoculated with homogenates from mice with centrosome autoantibodies, but not from centrosome autoantibody-negative animals (data not shown). The infectious agent was plaque-purified (Fig 6a-b), and then banded on renografin density gradients (1.19-1.20 g/cm³, Fig.6c).

Plaques induced by the isolated infectious agent were indistinguishable from those observed with spleen homogenates and were atypical in many ways. Plaque formation was unusually prolonged (7-10 days post-infection compared to most viruses, 1-4 days) and was consistently observed in a wide range of host cells (fibroblasts, lymphocytes and epithelial cells from human, monkey and mouse). Infection of cultured cells occurred without significant cell lysis (Fig. 6h), except in agar plaque assays where cells lost could not be replaced by the ingrowth of the surrounding monolayer. Several antibiotics prevented plaque formation (doxycyclin, ciprofloxacin, actinomycin D, Fig.7a, b) ruling out most viruses as infectious agents. The infectious agent appeared to be a membrane-bound, DNA-containing particle. It was unable to induce plaque formation when treated with organic solvents or nonionic detergents. Antibodies generated against renografin fractions in mice labeled DNA-containing subcellular particles at the margins of plaques when visualized by immunofluorescence microscopy (Fig. 6d-g). Electron microscopic

examination of plaques (Fig.8c) and pellets of peak gradient fractions (Fig.8d) revealed phenotypically homogeneous structures $\sim 0.45 \mu\text{m}$ in diameter that lacked cell walls and were closely apposed to the plasma membrane of host cells (Barile and Razin, 1979). Taken together, these features suggested that the agent was a mycoplasma.

Identification of the Infectious Agent as Mycoplasma. We used several approaches to confirm that the infectious agent was a mycoplasma. We obtained a monoclonal antibody specific for *Mycoplasma hyorhinis* variable lipoprotein (Vlp) (Rosengarten and Wise, 1990) and used immunofluorescence microscopy to show that it labeled the margins of plaques containing our infectious agent (compare Fig. 8a-b and Fig. 6d-g). A second, polyclonal anti-mycoplasma antibody also labeled small dense particles from our renografin fractions that co-labeled with the antibody generated by mice infected with renografin fractions. In another approach, we obtained *Mycoplasma hyorhinis* from a commercial source and demonstrated that it formed plaques with similar morphology and kinetics to those observed following inoculation with renografin fractions. The identity of the infectious agent as mycoplasma was definitively determined by DNA sequence analysis.

DNA was isolated from peak renografin gradient fractions, restriction-enzyme digested, cloned and sequenced. Sequences from most clones (74%, n=34) exhibited a high degree of homology to Mycoplasma genes including variable lipoprotein (vlp), 16S/23S rRNA intergenic spacer, pyruvate dehydrogenase, aminoacid permease and methylase (Fig. 9a-

c, Table II); the remaining clones had no significant homology to NCBI database sequences. Additional sequences were obtained by PCR analysis of DNA from our renografin-fractionated material. Several genes were amplified and again, all were most homologous to mycoplasma sequences including the 16S rRNA gene, the putative transcription elongation factor (tuf) and triosephosphate isomerase (tpi, Table II).

Sequences obtained both by random cloning and PCR analysis shared strongest homology with the porcine pathogen *Mycoplasma hyorhinis*. Because mycoplasmas are known contaminants of tissue culture cells, we performed additional tests to demonstrate that our fractions were derived from mouse material. We took precautions to specifically use mycoplasma-free cell lines available from ATCC and confirmed that they were mycoplasma-free by PCR using highly sensitive commercial primers (Stratagene) or those produced in our own laboratory (see Materials and Methods). Importantly, we showed that mycoplasma sequences could be amplified directly from spleens and lungs of mice that had spontaneously produced centrosome autoantibodies (above) but had never come in contact with tissue culture materials.

Mycoplasma Induces Centrosome Autoantibodies in Naive Mice. To demonstrate that mycoplasma was the causative agent of autoantibody production in mice, we delivered plaque-purified, gradient banded mycoplasma fractions to naive wild type mice and assayed for centrosome autoreactivity. Intranasal delivery consistently induced autoantibody production in over half the mice (Fig. 10 a-d, Table III). Oral delivery was

also effective while intraperitoneal or intravenous injection induced no autoantibodies, demonstrating that the mode or route of delivery was important for autoantibody production (Fig. 11). The efficacy of intranasal delivery of mycoplasma suggested that autoantibody development may require an acute infection, perhaps within mucosal tissues. To test for mycoplasma infection, we examined sera from intranasally infected and control mice for the presence of anti-mycoplasma antibodies by immunofluorescence staining of mycoplasma particles affixed to coverglass (above). All animals that produced centrosome autoantibodies following delivery of mycoplasma had detectable anti-mycoplasma antibodies in their sera. In contrast, animals that received control inoculum, showed no detectable signs of anti-mycoplasma antibody production. These and other results (below) suggested that infection by mycoplasma is required to induce centrosome autoantibody production.

Mycoplasma-Induced Centrosome Autoantibodies Can Be Prevented by Antibiotic Treatment. The ability to induce centrosome autoantibody production by infection with mycoplasma offered a unique opportunity to test whether autoreactivity could be inhibited by targeting mycoplasma with antibiotics. As expected, mice that received solution without the antibiotic ciprofloxacin mounted a strong autoantibody response (Fig. 12). However, pretreatment of mice with ciprofloxacin prior to mycoplasma infection inhibited autoantibody production ($P=0.0013$ Fig. 12). Centrosome autoantibodies were undetectable at all times examined (from 0 to 6 weeks post-infection) and were indistinguishable from uninfected mice. A low percentage of

infected, antibiotic-treated mice developed strong autoreactivity (~10%). Although we do not have an explanation for this result, it may be due to ineffective delivery of the antibiotic, incomplete bacteriostatic activity of the antibiotic in some mice, or other reasons.

The Centrosome Autoantibody Response Involves Isotype Switching and Amplifies to Include Additional Intracellular Targets. A more detailed analysis of centrosome autoreactivity in mice revealed that the mycoplasma-induced response was mainly of the IgG isotype (Fig.10f). Importantly, anti-centrosome IgMs were detectable by IgM-specific secondary antibodies in naive wild-type mice, and their titers do not increase following mycoplasma infection (Fig.10e). This suggests that the self-reactive B lymphocytes that contribute centrosome-reactive IgMs to the natural antibody repertoire of unimmunized mice, may respond to infection by class switching to high titer IgGs. The centrosome autoantibodies are of the IgG1 and IgG2b subclasses and appear as early as 2-3 weeks post-infection in some animals and are well established by 6-8 weeks in all animals. These anti-centrosome IgGs remain the only detectable autoantibody specificity for at least 8 weeks post-infection (Fig 10 a-d).

A unique feature of the murine autoantibodies produced by mycoplasma infection was their exclusive centrosome-specificity. This raised the hypothesis that centrosomes may initiate an autoantibody response that subsequently spreads to include reactivity to other intracellular organelles. This phenomenon, known as antigenic spreading, has been

implicated in the generation of complex multi-target autoantibody responses in autoimmune diseases (Craft and Fatenejad, 1997). To address this issue, we examined mice at later times after mycoplasma infection to determine whether additional autoantibodies were generated. Beginning at approximately 12 weeks post-infection, autoantibodies to another intracellular structure were detected in infected mice. Immunofluorescence co-localization of mycoplasma-induced autoantibodies with antibodies to calnexin (Fig. 13 a-c) and to the Binding protein of the endoplasmic reticulum (Bip, data not shown) (Linnik and Herscovitz, 1998; Nelson et al., 1998), demonstrated that this structure was the endoplasmic reticulum (ER). Autoantibodies to the ER developed specifically in infected mice, they had lower titer than anti-centrosome autoantibodies and they were of IgM isotype only (Fig. 13d-e). These results demonstrate that mice elicit complex autoantibody responses following mycoplasma infection.

D. Discussion

These experiments show that mycoplasma infection causes the development of scleroderma-like anti-centrosome autoantibodies in mice. They represent a first demonstration of a complex autoantibody response initiated by centrosome autoantigens, which spreads to include additional autoantigens and is treatable by the antibiotics that eradicate the bacterial infection. These results also imply that the initial loss of tolerance to centrosome antigens precedes the development of the more complex autoantibody response to numerous autoantigens, which occurs in scleroderma.

One aspect of autoantibody development in mycoplasma-infected mice unique from other experimental systems is that the initiating target antigens are exclusively centrosomal. This is in contrast to other studies, which describe infection-induced autoantibodies with multiple specificities characteristic of systemic lupus erythematosus (Flaegstad et al., 1988; Olding et al., 1976; Sangster et al., 2000). These studies may either describe a polyclonal B cell activation or not distinguish an initiating autoantigen.

The initiation of autoreactivity in scleroderma by centrosome antigens is supported by a prospective study of autoantibody development in a large cohort of individuals at risk for occupational scleroderma. A subset of these individuals developed Raynaud's syndrome, a prodromal scleroderma feature, and progressed to suspected, then certain, scleroderma. At the earliest stages of disease, autoantibodies to mitotic spindles were the only detected specificities (Conrad and Mehlhorn, 2000). Prior to this prospective study, patients with Raynaud syndrome or scleroderma having only autoantibodies to centrosomes have been described in a number of reports (Doxsey et al., 1994; Gentric et al., 1990; Mack et al., 1998; Moteki et al., 1991; Osborn et al., 1982). It is conceivable that autoantibodies to mitotic spindles or centrosomes are masked at later disease stages by the development of additional autoantibody specificities (Gavanescu et al., 1999).

Autoantibodies to nuclear antigens may be spontaneously generated in normal mice by altering the signalling threshold of B cells. B cells from mice transgenic for the human

activating receptor CD19, which have a lowered signalling threshold, develop only autoantibodies to the centriole, which is the centrosomal core, when the transgene is expressed at low levels. Higher levels of transgene expression trigger the development of a homogeneous nuclear staining pattern in addition to centriolar (Sato et al., 2000). These data imply that autoreactive centrosome-specific B cells have less stringent activation requirements compared to other autoreactive B cells and are activated first during scleroderma-like autoantibody responses.

Individuals with *M. pneumoniae* infection frequently develop both recurring and rare autoantibody specificities to: brain, heart and muscle, erythrocyte I antigen, intermediate filaments and mitotic spindle (Cassell et al., 2001-2002). The oligoclonal IgM autoantibody responses to endoplasmic reticulum antigens that result upon infection in the experiments of this report are very similar to the IgM autoantibodies specific for the I antigen of red blood cells frequently induced by *Mycoplasma pneumoniae* (Cassell et al., 2001-2002). Autoantibodies to mitotic spindles, centrosomes or centrioles are very rare in association with infection, in patients infected with *Mycoplasma pneumoniae*, EBV or HIV (Auer-Grumbach and Achleitner, 1994; Cimolai et al., 1994; Gentric et al., 1991; Huidbuchel et al., 1991; Lind et al., 1988; Moteki et al., 1991). Although anecdotal, these correlations support the results of our experiments.

The mechanism by which mycoplasma infections initiate immune responses that ultimately result in autoantibody production is currently unknown. Mycoplasmas present

a variety of structures capable to engage both innate and adaptive components of the immune system. Mycoplasma superantigens have been described and may account for polyclonal activation of autoreactive B and T cells with anti-centrosome and anti-ER specificities. The mycoplasma macrophage-activating lipopeptide (MALP) may be able to stimulate B lymphocytes that also express Toll-like 2 receptors (Lien et al., 1999; Seya and Matsumoto, 2002; Takeuchi et al., 2000). Toll-like receptors can initiate polyclonal autoantibody responses that may include anti-centrosome and anti-ER specificities. However, dual activation of Toll-like receptors and specific immunoglobulin receptors has been recently demonstrated to initiate specific autoantibody responses and is most likely to account for the oligoclonal anti-centrosome and anti-ER autoantibodies described in this work (Leadbetter et al., 2002).

Autoantibody responses initiated by centrosome antigens may be amplified by the recruitment of additional specificities. Such amplification results when the response to the initiating epitope expands to encompass epitopes within the same or within adjacent molecules; this process, defined as epitope spreading has been observed frequently following peptide immunization (Bockenstedt et al., 1995; Craft and Fatenejad, 1997). As the expansion of the response to immunodominant epitopes progresses, isotype shifts result, from IgM to other isotypes (Scofield et al., 1996). IgM autoantibodies may be initially recruited into the response from the natural antibody repertoire. Autoreactive IgM of the natural antibody repertoire have a dual role in the unimmunized host, of providing protection from bacteria and clearing altered self-antigens that result from

lipoprotein oxidation or cellular apoptosis (Briles et al., 1982; Shaw et al., 2000). Similarly, anti-centrosome IgM may be part of the natural antibody repertoire of normal mice and have the role to provide protection against mycoplasmas, while exacerbations of the numbers of infectious particles during acute mycoplasma infections may trigger class-switching to anti-centrosome IgG. As the response to the initiating epitope progresses to IgG isotype, epitope spreading may involve additional epitopes on ER antigens by recruiting new autoantibody specificities of IgM isotypes.

Lastly, initiation of scleroderma-like autoimmune responses by bacterial pathogens suggest that antibiotic treatment at early stages of scleroderma may have an ameliorative effect. Intriguing clinical remissions of scleroderma with antibiotics effective on mycoplasmas have already been reported (Le et al., 1998) (Over and Bucknall, 1998).

Table I. Prevalence of Anti-Centrosome Autoantibodies in Mouse Colonies

Method	Colony	Proportion centrosome autoantibody-positive
Immunoblotting to recombinant centrosome protein pericentrin	A*	0/17 (0%)
	B*	0/5 (0%)
	C*	1/6 (16%)
	D*	5/20 (25%)
Immunofluorescence to centrosome, co-labeled with marker antibody to pericentrin	E*	55/154 (35%)
	F*	1/6 (16%)

*Academic and commercial mouse colonies listed in Materials and Methods

Fig 5. Induction of centrosome autoantibody development. Autoantibodies can be transmitted to naive mice housed with autoantibody-positive mice. Immunoblots of recombinant pericentrin (a) or centriolin (clone name CP140, b), using ten-fold serially diluted sera from two mice that were naive at the initiation of the experiment (lanes 1-6 blots a, b), and when placed in contact with an anti-centrosome antibody-positive mouse (lanes 7-12 blots a, b), but develop autoantibodies to pericentrin and centriolin (lanes 13-18 blots a, b) 2 weeks after being housed in contact with sero-positive mice. The result is representative of 2 experiments.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

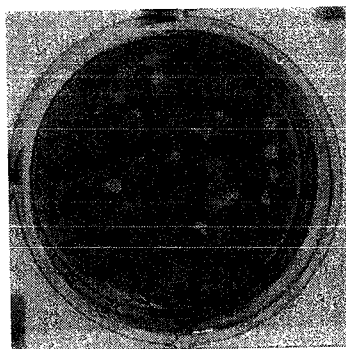
a.

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

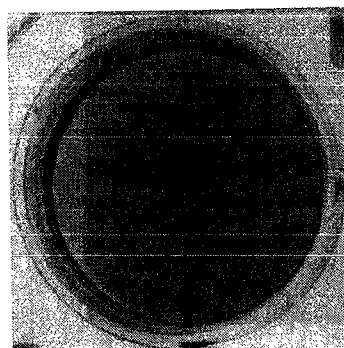
b.

Fig.6. Isolation and characterization of a plaque-forming infectious agent. Infected inoculi form plaques in a concentration-dependent manner when serially diluted and inoculated on cultured Vero cell monolayers (a) while no plaques are seen in cells inoculated with culture media alone (b). Banding and enrichment of infectious agent in renografin gradients. Titers are determined by plaque assay (diamonds, \log_{10} titer in plaque-forming units, p.f.u.). Refractive index (squares) of renografin gradient fractions (c). Antibodies developed by mice immunized with the infectious agent detect cell-associated antigen at the margin of a plaque(d). Dapi-staining nuclei of cell monolayer surrounding a plaque(e). Spherical, particulate antigenic structures within infected cells (f) co-localize with DNA-containing particles labeled by dapi (g). Titer of infectious agent increases and plateaus in infected cultures at similar rates irrespective of whether high or low multiplicity of infection (m.o.i., same number of p.f.u for squares= 10^5 cells infected /well and diamonds= 10^4 cells infected/well) (h).

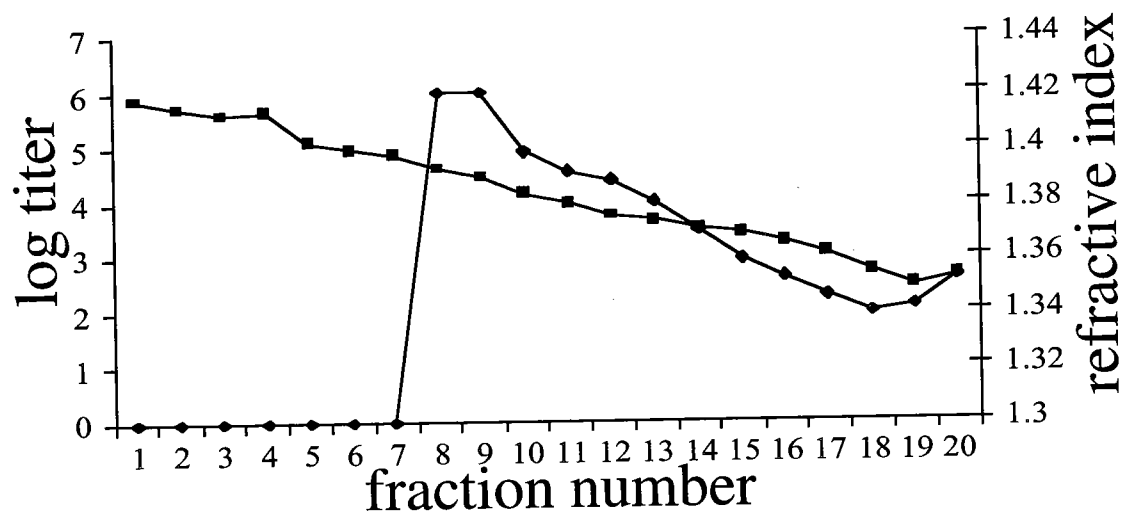
6a.



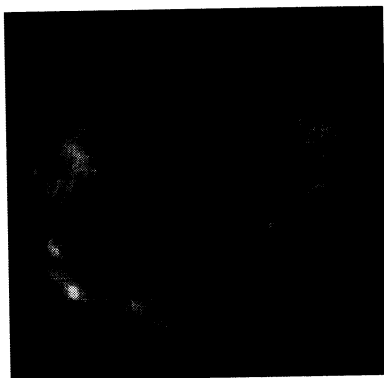
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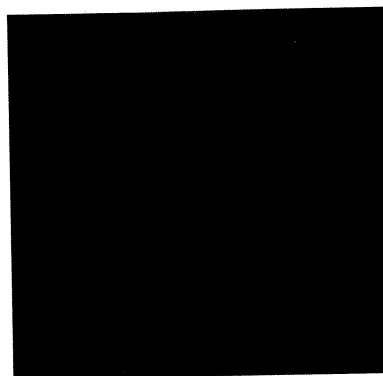
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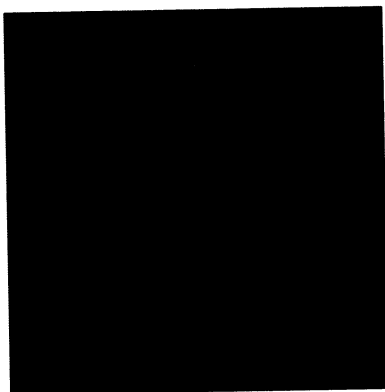
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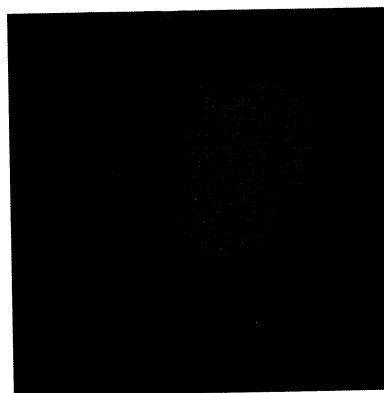
6e.



6f.



6g.



6h.

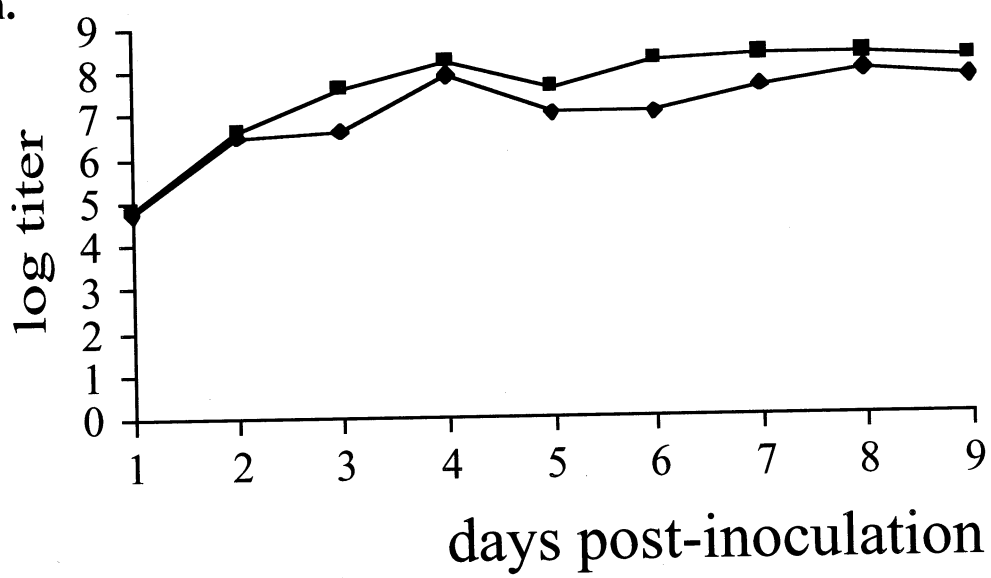
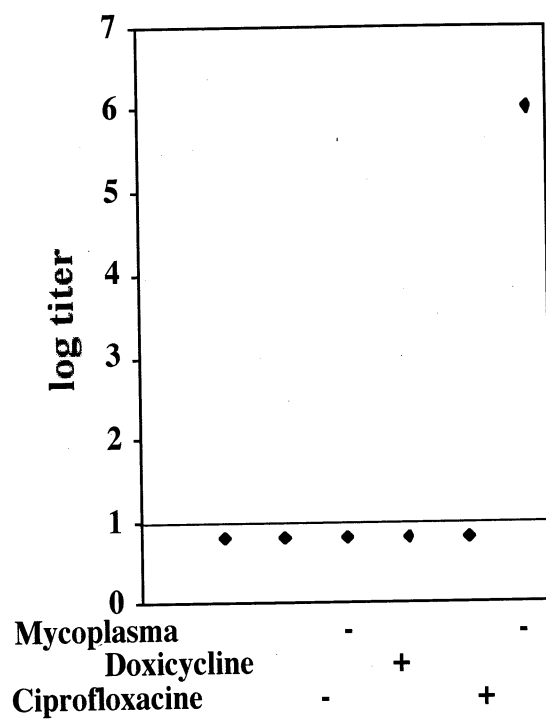


Fig. 7. Infectious agent sensitivity to antibiotics. The murine infectious agent is sensitive to treatment with bactericidal antibiotics (doxycyclin and ciprofloxacin, **a**). Plaque formation is abolished in antibiotic-treated, but not in untreated cultures; uninfected monolayers do not develop plaques (assay sensitivity, 10 plaque-forming units/ml). The growth of the murine infectious agent is inhibited by treatment with actinomycin D, an antibiotic that inhibits DNA-dependent RNA synthesis (**b**). Infectious agent titers increase 100 fold over 24 hours of infection (monolayer of 293T cells, ●), but no growth is detected when infected cultures are treated with actinomycin D (▲). Herpes simplex virus I is used as a positive control, showing plaque-formation in the absence (Δ) but not in the presence (×) of actinomycin D. Sindbis virus growth has the same kinetics independent of the presence (■) or absence (◆) of actinomycin D. Act.D was added 6 hours post-infection for the rapidly growing Sindbis virus and 12 hours post infection for the murine infectious agent and the herpes virus; addition of act. D at 12h defines the starting point of the time course. Assay detects 10 pfu/ml.

7a.



7b.

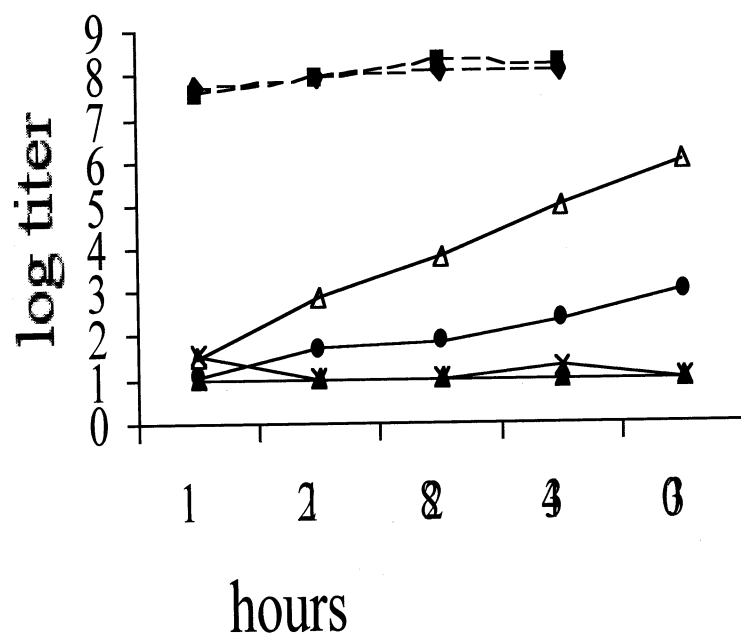
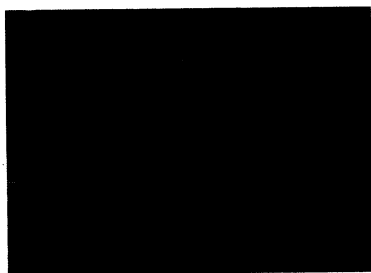
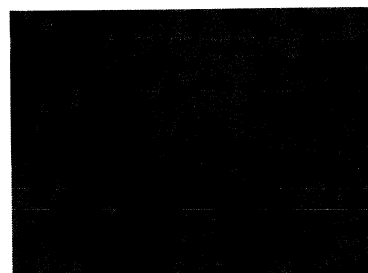


Fig. 8. Identification of the plaque-forming infectious agent as a mycoplasma using a monoclonal antibody specific for a unique *Mycoplasma hyorhinis* surface antigen (**a**). Nuclei of Vero cells surrounding the plaque are stained with dapi (**b**). Electron microscopy reveals that the infectious agent associated with plaques (**c**) and present in peak gradient fractions (**d**) has a morphology consistent with that of a mycoplasma.

8a.



8b.



8c.



8d.

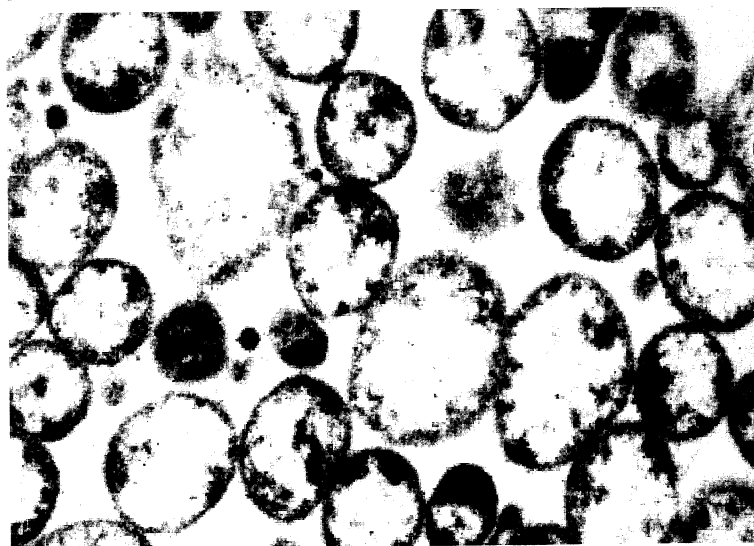


Table II. Summary of Sequences of the Murine Mycoplasma Isolate

Gene name (symbol)	Detection method	Sequence size (bp)	Number of sequences	Homology to pig M. hyorhinis	Homology to bacteria <u>sequence</u>	other <u>protein</u>
Variable lipoprotein (vlp)	Random cloning	191	4	100%	none	none
16s/23s rRNA spacer	Random cloning	408	2	95%	Mycoplasma hominis 88%	NA ^a
16s rRNA	PCR	354	8	100%	Mycoplasma moatsii 97%	NA ^a
Putative transcription elongation factor (tuf)	PCR	883	8	99%*	Mycoplasma pulmonis 79%	Mycoplasma pulmonis 78%
Putative methylase	Random cloning, PCR	185	8	100%	none	Mycoplasma mycoides 59%
Putative pyruvate dehydrogenase	Random cloning, PCR	210	4	100%	none	Mycoplasma pulmonis 31%
Putative permease	Random cloning, PCR	130	12	100%	none	Mycoplasma pneumoniae 19%
Trioso phosphate isomerase (tpi)	PCR	512	8	100%	Mycoplasma pneumoniae 39%	Mycoplasma pulmonis 56%

*Silent and non-silent point mutations in tuf gene

^aNA, not applicable

Fig. 9. Pathogen identification by genome sequence. Comparison of mycoplasma phylogenetic and taxonomic markers (16s rRNA gene, **a**; and the 16s/23s rRNA intergenic spacer, **b**). The murine (M) mycoplasma (M-16s and M-16s-23s) is highly homologous to pig (P) *Mycoplasma hyorhinis* (specifically amplified P-16s or 16s-23s spacer of the NCBI database, accession number AF121891). Comparison of the variable lipoprotein (vlp) gene encoding a surface antigen (**c**) between mouse *Mycoplasma* isolates (M-vlp) and pig *Mycoplasma hyorhinis* NCBI data base sequence AF193880 reveals high sequence homology between the two isolates .

9a.

M-16s GTTAAGTCCTGCAACGAGCGCAACCCCTTTTCTTTAGTTACTAATATTAAGTTAAGGACTCTAGAGATACT
P-16s GTTAAGTCCTGCAACGAGCGCAACCCCTTTTCTTTAGTTACTAATATTAAGTTAAGGACTCTAGAGATACT

M-16s GCCTGGGTAAACGAGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCTCTTACGAGTGGGGCAACACAC
P-16s GCCTGGGTAAACGAGGAGGAAGGTGGGGACGACGTCAAATCATCATGCCTCTTACGAGTGGGGCAACACAC

M-16s GTGCTACAATGGTCGGTACAAAGAGAAGCAATATGGTGACATGGAGCAAATCTCAAAAAACCGATCTCAG
P-16s GTGCTACAATGGTCGGTACAAAGAGAAGCAATATGGTGACATGGAGCAAATCTCAAAAAACCGATCTCAG

M-16s TTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGCTACGCTAC
P-16s TTCGGATTGAAGTCTGCAACTCGACTTCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGCTACGCTAC

M-16s GGTGAATACGTTCTCGGGTTTTGTACACACCGCCCGTCACACCATGGGAGTTGGTAATGCCCAAAGTCGG
P-16s GGTGAATACGTTCTCGGGTTTTGTACACACCGCCCGTCACACCATGGGAGTTGGTAATGCCCAAAGTCGG

M-16s TTT
P-16s TTT

9b.

M-16s-23s ATTAAGCTATGCAGTCGGAACGTGGGTGATAACG]-TCCACGCT]CGGAGGGAAACAACCCAGATCGTCAG
AF121891 ATTAAGCTATGCAGTCGGAACGTGGGTGATAACGTTCCACGCT]GCGAGGGAAACAACCCAGATCGTCAG

M-16s-23s CTAAGGTCCCAAAATTGTGTTAAGTGAGAAAGGTTGTGAGATTTTATAA]CA]CTAGGAAGTTGG]CTTAG
AF121891 CTAAGGTCCCAAAATTGTGTTAAGTGAGAAAGGTTGTGAGATTTTATAA]CA]CTAGGAAGTTGG]CTTAG

M-16s-23s AAGCAGCCACCTTTT]-AAAGAGTG]GTAATTGCTCACTAGTCAA]GAGAT]CTTGGCCCAATAATGTAACGG
AF121891 AAGCAGCCACCTTTT]-AAAGAGTG]GTAATTGCTCACTAGTCAA]GAGAT]CTTGGCCCAATAATG]-AACGG

M-16s-23s GACTCAAAACACAATACCGAAGCTACGGGCACATTATGTGCG]-TTAGGAGAGCG]-TTTTAATTTCTT]GAA
AF121891 -]ACTCAAAACACAATACCGAAGCTACGGGCACATTATGTGCG]TTAGGAGAGCG]TTTTAATTTCTT]TAA

M-16s-23s -GTCAG]ACCGT]G]-GGACTGGTG]AGAGATTAAAAGTGAGAAATGCCGGCATGAGTAACGATTGGAAGTGA
AF121891 -GTCAG]ACCGT]T]AAGGACTGGTG]AGAGATTAAAAGTGAGAAATGCCGGCATGAGTAACGATTGGAAGTGA

M-16s-23s GAATCTTCGACGCCTATTGGGAAAGGTTTCCTGGGCAAGGTTTCGTCACCCAGGGTTAGTCAG
AF121891 GAATCTTCGACGCCTATTGGGAAAGGTTTCCTGGGCAAGGTTTCGTCACCCAGGGTTAGTCAG

9c.

M-vlp AAAA CTGAAAACACACAACAAAGTGAAGCTCCAGGAACAAAACTGAAAACACACAACAAAGTGA
AF193880 AAAA CTGAAAACACACAACAAAGTGAAGCTCCAGGAACAAAACTGAAAACACACAACAAAGTGA

M-vlp AGCTCCAGGAACAAACACTGGAAAACAAACAACTAGTGAATCAAATTCGGATCATCTACAGGTA
AF193880 AGCTCCAGGAACAAACACTGGAAAACAAACAACTAGTGAATCAAATTCGGATCATCTACAGGTA

M-vlp GTCAAGCAGGAACAACTACAAATACAGGTTAGTGGATCAAATTCGAAATCAGGAATGAATTC
AF193880 GTCAAGCAGGAACAACTACAAATACAGGTTAGTGGATCAAATTCGAAATCAGGAATGAATTC

Table III. Mycoplasma-Infected Mice Develop Autoantibodies to Centrosomes

Experiment*	Mycoplasma infection	Numbers	Proportion centrosome autoantibody-positive ^b	Statistical significance ^c
1	none	n = 4	0% (n = 0)	<i>P</i> < 0.005
	5 x 10 ⁶ p.f.u.	n = 12	50% (n = 6)	
2	none	n = 6	0% (n = 0)	<i>P</i> < 0.05
	5 x 10 ⁶ p.f.u.	n = 8	62.5% (n = 5)	
3	none	n = 8	0% (n = 0)	<i>P</i> < 0.001
	5 x 10 ⁶ p.f.u.	n = 7	71.4% (n = 5)	
4	none ^a	n = 5	0% (n = 0)	<i>P</i> < 0.05
	5 x 10 ⁶ p.f.u.	n = 12	75% (n = 9)	

* Experiments 1-4 performed in Balb.c/SvJ mice having the lowest prevalence of spontaneous autoantibody development

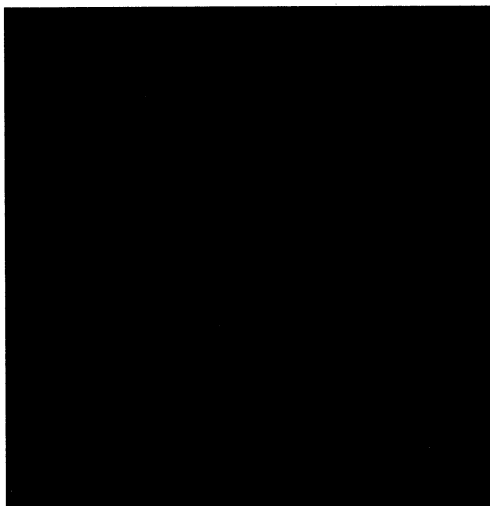
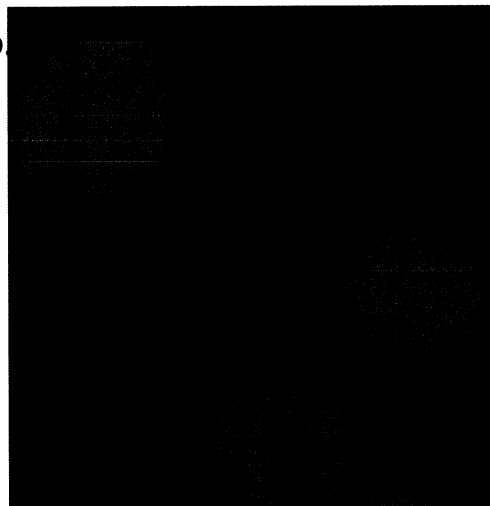
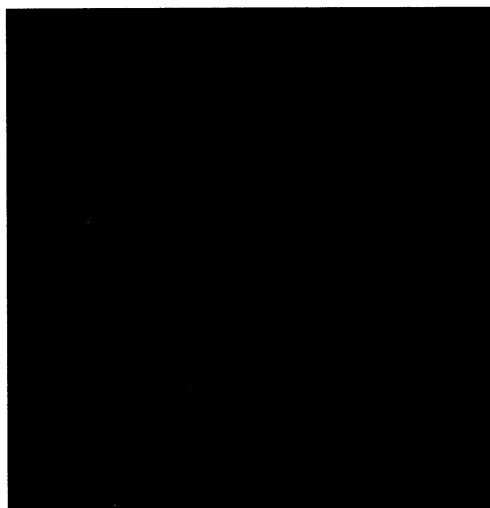
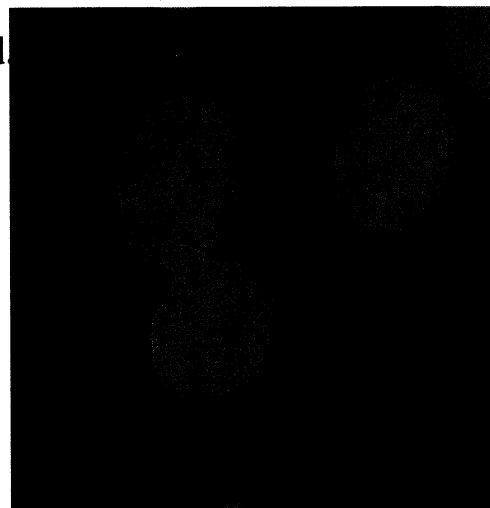
^a Control mice treated with antibiotic in exp. 4

^b An arbitrary assay cut-off value was chosen for a 3-4 fold change in titer

^c The Student t-test, two-tailed with unequal variances, was used for statistical comparison between infected and uninfected mouse groups

Fig.10. Mycoplasma-infected mice develop anti-centrosome autoantibodies.

Immunofluorescent staining of centrosomes by anti-centrosome autoantibodies in sera of mycoplasma-infected mice (a). Specific antibody to centrosome protein pericentrin identifies centrosomes (green) adjacent to dapi-staining cell nuclei(blue, b). In the absence of autoantibodies in mouse sera(c), centrosomes are detected only by the pericentrin antibody in the vicinity of dapi-staining nuclei (d). Anti-centrosome IgM autoantibody titers as detected by immunofluorescence do not differ significantly in mycoplasma-infected and uninfected mice 21 weeks post-infection ($p=0.765$, $p>0.05$)(e). Mycoplasma-infected mice develop significantly higher titers of anti-centrosome IgG autoantibodies 21 weeks post-infection in contrast to uninfected mice ($p=0.001625$) (f). Mouse autoantibody titers were determined by endpoint titration and are expressed as the reciprocal of the serum dilution. Each point represents the result for an individual mouse. Results are representative of at least 4 independent experiments.

10a.**10b.****10c.****10d.**

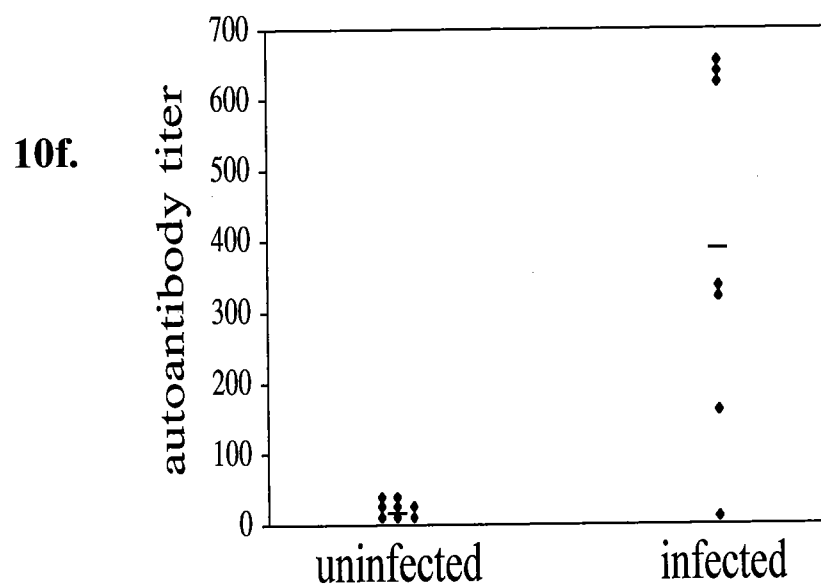
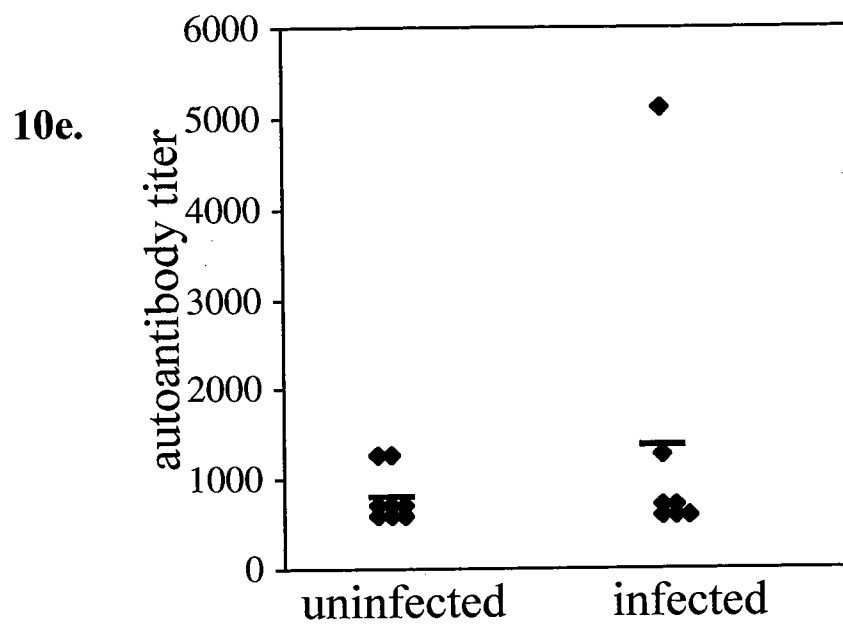


Fig 11. Oral and intranasal, but not intravenous and intraperitoneal mycoplasma infection induces the development of autoantibodies to the centrosome protein pericentrin. Immunoblots of recombinant pericentrin: two 4-fold serially diluted serum samples assayed for each mouse infected oral (**a**, lanes 1-8), intranasal (**a**, lanes 9-16), intravenous (**b**, lanes 1-8) and intraperitoneal (**b**, lanes 9-16). Results are representative of 3 experiments.

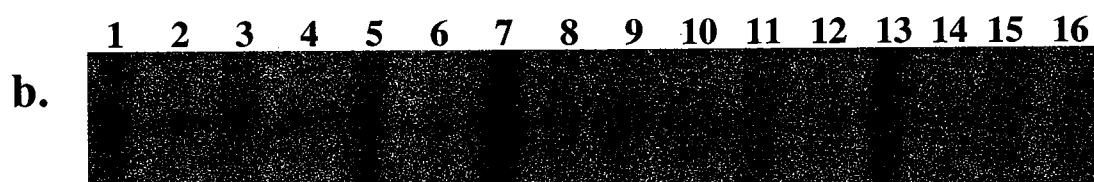
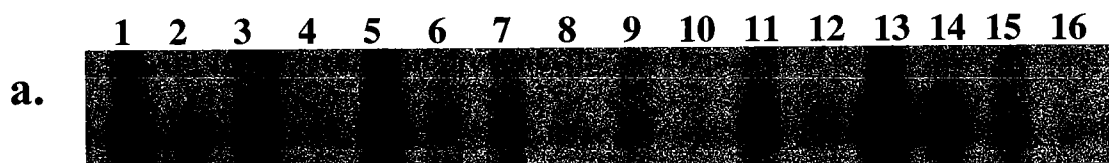
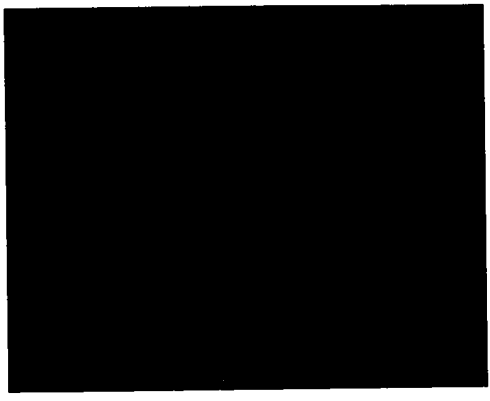


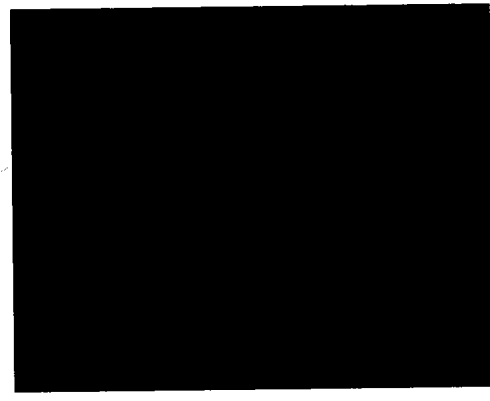
Fig.12. Antibiotic treatment prevents the development of centrosome autoantibodies in mycoplasma infected mice. When mice are treated with the bacteriostatic antibiotic ciprofloxacin, autoantibodies to centrosomes do not differ significantly in titer in mycoplasma infected as compared to uninfected mice ($P=0.6064$). Positive control mice, that are not treated with antibiotic but infected with mycoplasma, develop significantly higher autoantibody titers as compared to antibiotic-treated mice ($P=0013$).

Fig.13. The autoantibody response amplifies to involve additional, endoplasmic reticulum-associated autoantigens. Nine-eleven weeks after observing centrosome autoreactivity in mycoplasma-infected mice, autoantibodies to a cytoplasmic antigen were detected by IF (**a**). They co-localize with the endoplasmic reticulum protein calnexin (**b**); cell nuclei are stained with dapi (**c**). Autoantibodies to the ER-associated autoantigen have significantly higher titers in infected as compared to uninfected mice ($P=0.005206$) (**d**). To compare titers of anti-centrosome antibodies (uninfected anti-centrosome: UI-C, infected anti-centrosome: I-C) with titers of antibodies to the ER-associated autoantigen (uninfected anti-ER: UI-ER, infected anti-ER: I-ER) we used a pan-specific antibody raised against immunoglobulin heavy and light chains. At any time post-infection autoantibodies to the ER-associated autoantigen have lower titers compared to anti-centrosome autoantibodies. Results are representative of 3 independent experiments. Autoantibodies to the ER-associated autoantigen are of IgM isotype and have significantly higher titers in infected as compared to uninfected mice ($P=0.0059$) (**e**).

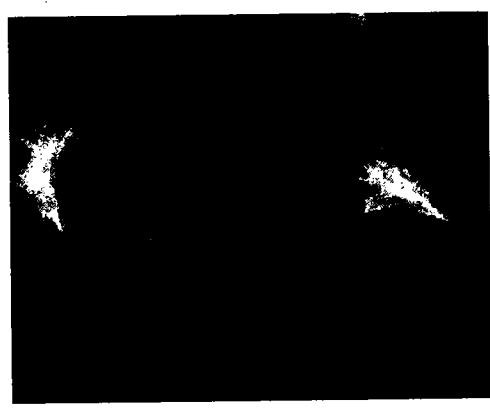
13a.



13b.



13c.



CHAPTER IV

CONCLUSIONS, DISCUSSION AND FUTURE DIRECTIONS

A. Conclusions

1. **Novel Methodology.** These studies developed sensitive and specific assays to determine the prevalence of anti-centrosome autoantibodies in patients with scleroderma.
2. **Autoreactivity to Centrosomes in Scleroderma.** Importantly, this analysis shows that centrosome antigens are major scleroderma autoantigens and are at least ten-fold more prevalent in patients than had been reported previously (Tuffanelli et al., 1983). Only a small percentage of normal individuals and patients with other connective tissue diseases have centrosome autoreactivity.
3. **Scleroderma Diagnosis.** Among scleroderma patients with centrosome autoreactivity, this work defines a subset that develop anti-centrosome antibodies, in the absence of antibodies against other autoantigens diagnostically found in scleroderma, such as topoisomerase I, centromeres etc. In these patients, anti-centrosome antibodies represent the sole class of autoantibodies that is detectable. Taken together, these data suggest that anti-centrosome autoantibodies may represent a new diagnostic tool in scleroderma (Fauci, 2001-2002).
4. **New Model.** These studies induce experimentally the development of anti-centrosome autoantibodies in mice. A murine model was developed to study infection-induced autoantibody production and this model was used to obtain information on:

5. **Class-switching** of centrosome autoantibodies from IgM to IgG isotypes and the subsequent recruitment of new autoantibody specificities of IgM isotype.
6. **Spreading** of centrosome-specific autoreactivity to recruit additional, endoplasmic reticulum autoantigens.
7. **Murine Mycoplasma.** These experiments isolate and characterize a murine mycoplasma able to induce autoantibody production upon infection of mice and provide the opportunity to modify bacterial properties to address the mechanisms of autoantibody development.

These results establish that centrosomes are major targets of autoantibodies in scleroderma and also provide evidence that mycoplasmas trigger autoreactivity to centrosomes. The possible role of mycoplasmas as cofactors in the pathogenesis of human autoimmune diseases is discussed below.

B. Discussion

Mycoplasmas Induce Autoantibodies. Numerous correlations in human patients support a link between mycoplasma infection and the development of autoantibodies. Individuals with *M. pneumoniae* infection can develop several autoantibody specificities, including autoantibodies to: brain, heart and muscle; erythrocyte I antigen; intermediate filaments; mitotic spindle (Cassell et al., 2001-2002).

Mycoplasma-induced IgM autoantibodies specific for the I antigen of red blood cells are able to trigger a self-limiting autoimmune hemolytic anemia (Cassell et al., 2001-2002). Interestingly, similar oligoclonal IgM autoantibody responses to endoplasmic reticulum antigens that result upon mycoplasma infection are described in this dissertation. In addition, our results are supported by descriptions of autoantibodies to centrosomes or mitotic spindles in a few case reports of human infections with *Mycoplasma pneumoniae* (Auer-Grumbach and Achleitner, 1994; Cimolai et al., 1994; Lind et al., 1988).

Mycoplasmas Induce Chronic Inflammatory Disease. Mycoplasmas are good candidates for co-factors in the development of rheumatic diseases. Mycoplasmas have been incriminated in the development of human arthritis. An important basis for this association is the fact that mycoplasmas trigger sterile arthritis in many animal species, including mice and rats (*Mycoplasma arthritidis*), cattle (*Mycoplasma bovis* and *Mycoplasma mycoides*), goats and sheep (*Mycoplasma agalactiae* and *Mycoplasma mycoides*), fowl (*Mycoplasma gallisepticum* and *Mycoplasma synoviae*) and pigs (*Mycoplasma hyorhinis* and *Mycoplasma hyosynoviae*) (Tully and Whitcomb, 1979).

In rheumatoid arthritis patients, several studies reported the detection of mycoplasma nucleic acid in the affected synovia but the results require further substantiation (Razin et al., 1998). Because mycoplasmas are commensal organisms and are frequently detected in healthy individuals as well, their role as etiologic triggers of arthritis would require the consistent detection of mycoplasmas in the chronic inflammatory lesion, ideally in association with cells that can be incriminated as targets or effectors of an immune attack.

Definitive proof of mycoplasma-induced arthritis development would be provided by the demonstration in an animal model of the pathogenic mechanism initiated by mycoplasma infection that results in a phenotype identical to human rheumatoid arthritis. The work described in this dissertation isolated a mycoplasma capable to induce in an animal host connective tissue disease features other than arthritis, substantiating an additional link between mycoplasma infection and autoimmunity.

Koch's Postulates in Mycoplasma Infections. Mycoplasmas are considered commensal organisms, acquired in utero, during or immediately after birth, that have low pathogenic potential in healthy humans or animals (Baseman and Tully, 1997; Cassell, 1998). Mycoplasma pathogenicity exacerbates in immunosuppressed individuals, including premature low-birth weight infants, patients with hypogammaglobulinemias of different etiologies, AIDS patients and patients receiving immunosuppressive drugs (Baseman and Tully, 1997; Cassell, 1998). Under these circumstances, an acute mycoplasma infection may result either from the exacerbation in numbers of endogenous mycoplasmas, or from de novo infection. Mycoplasmas evade immune defenses and antibiotic treatment by multiple mechanisms and are not eradicated after acute infection. Therefore, the concept of chronic mycoplasma infection would rather refer to the persistence of mycoplasma-induced pathology, than to the mere presence of detectable mycoplasmas. Also, demonstrating Koch's postulate concerning the ability of mycoplasmas to induce disease would need to reproduce the conditions that confer commensal mycoplasmas a pathogenic potential. Our results show that experimental inoculation of high numbers of

viable mycoplasmas is sufficient to induce in healthy mice scleroderma-like autoantibody responses. The development of a full-blown scleroderma disease phenotype may require not only exacerbations of mycoplasma numbers, but possibly also host immunosuppression. Also, fulfillment of the last of Koch's postulates, requiring the reisolation of the mycoplasma from autoantibody-positive mice, would need to employ an in vitro tagged, transposon-mutagenized mycoplasma, that could be distinguishable from endogenous mycoplasmas.

A Novel Murine Mycoplasma. The murine mycoplasma has high genome sequence homology to the pig pathogen *Mycoplasma hyorhinis*. The isolation of *Mycoplasma hyorhinis* from mice has not been reported previously. By this criterion, we have isolated a novel murine mycoplasma. These findings expand the information on the natural habitat of *Mycoplasma hyorhinis*. In support of this result, several mycoplasma species, including *M. hyorhinis*, have been previously isolated from more than one animal host (Tully and Whitcomb, 1979). Interestingly, infection with *Mycoplasma hyorhinis* does not induce any overt pathology in a number of mouse strains, but triggers pneumonitis and arthritis in pigs. Mycoplasmas may be commensal in one animal host, while being pathogens in another animal species. The zoonotic development of mycoplasma-induced arthritis is an intriguing possibility.

The development of scleroderma-like autoantibodies to centrosome antigens upon mycoplasma infection indicates a possible role for mycoplasmas as co-factors in the

development of scleroderma. A link between mycoplasma infection and scleroderma development can be investigated by taking several approaches.

C. Future Directions

The results presented in this dissertation provide the basis for several future studies.

1. Define Additional Infectious Agents that May Induce Centrosome Autoantibodies. It is important to establish whether the development of autoantibodies to centrosomes can be initiated only by mycoplasmas, generally by bacteria or by any infectious agent including viruses or parasites. It is possible and likely that other mycoplasmas, infecting mice or other animal species, have the ability to induce anti-centrosome antibodies. It is also possible that other infectious agents including viruses are capable to initiate an anti-centrosome antibody response. Viruses may have the ability to directly induce the development of scleroderma-like autoantibodies or may alternatively induce a host immunosuppression that would allow the exacerbation of endogenous mycoplasmas, and these in turn may induce autoantibody development (Naniche and Oldstone, 2000; Rouse and Horohov, 1986; Schneider-Schaulies et al., 2001). Such hypotheses can be directly tested in the murine model developed in our study. An intermediate role of endogenous mycoplasmas in the development of virus-induced anti-centrosome autoantibodies can be distinguished by long-term antibiotic treatment of virally infected mice. Antibiotic treatment is expected to prevent autoantibody development induced by endogenous bacteria. The outcomes of this set of

experiments provide important insights into the opportunity of an antibiotic therapy for scleroderma. We have already shown that antibiotic treatment prior and during mycoplasma infection of mice abrogates anti-centrosome autoantibody development. However, if infection with viruses or parasites can also induce scleroderma-like autoantibodies, antibiotic treatment of scleroderma would unlikely be curative. The findings of the proposed experiments would complete the information provided by a few case reports describing remissions of scleroderma upon treatment with the antibiotics minocycline and ciprofloxacin, which have bacteriostatic effects on mycoplasmas (Le et al., 1998; Over and Bucknall, 1998).

2. Detect Tissue-Specific Autoantigens. A complete characterization of mycoplasma-induced autoantibody responses would include the detection of possible organ-specific autoantibodies. *Mycoplasma pneumoniae* has been associated with organ-specific autoantibody development in patients (Cassell et al., 2001-2002). Several other infectious agents have been shown to induce experimentally organ-specific autoantibodies (Bartholomaeus et al., 1988; Olding et al., 1976). Autoantibodies to tissue-specific autoantigens have been incriminated in organ-specific autoimmune pathogenesis. In mice deficient in the *aire* gene, which directs ectopic thymic expression of tissue-specific antigens and may promote thymic tolerance to self, organ-specific autoantibody development accompanies lymphocytic infiltration that results in disease (Anderson et al., 2002). The histological assay of mycoplasma-induced organ-specific autoantibody responses could employ tissue substrates from alymphocytic mice deficient in the

recombinase-activating gene (Rag), to prevent interference with endogenous immunoglobulins. The detection of organ-specific autoantibodies that occur as a consequence of mycoplasma infection is a prerequisite in investigating their possible potential to trigger scleroderma-like pathology (Section 4, below).

3. Generate Pathogenic Infection-Induced Autoantibodies. Infection-induced autoreactive B lymphocytes are normally short lived and destined to undergo apoptosis (Ray et al., 1996). Therefore, prolonging autoreactive B cell survival with the accumulation of such cells above a critical numerical threshold may result in autoantibody-mediated pathology. BLyS is a protein that has been recently demonstrated to promote autoreactive B cell survival and autoantibody development (Gross et al., 2001; Khare et al., 2000). Therefore, injection of recombinant BLyS in conjunction to intranasal mycoplasma infection may greatly enhance the amplitude of the infection-induced autoantibody response and possibly initiate pathology.

In addition, BLyS has been shown to promote isotype switching in the absence of T cells (Litinskiy et al., 2002). In turn, isotype switching alters the ability of autoantibodies to initiate tissue destruction by influencing their binding to Fc receptors and complement (Azeredo da Silveira et al., 2002). We have shown that mycoplasma infection induces in wild-type mice the development of class-switched autoantibodies of IgG1 and IgG2b isotypes, but not scleroderma-like pathology. BLyS-treatment that would accompany mycoplasma-infection may confer infection-induced autoantibodies additional isotypes at high titers, some of which may induce scleroderma-like pathogenesis.

4. Test the Pathogenic Potential of Infection-Induced Autoantibodies. It would be of tremendous interest to establish whether the autoantibodies that develop upon mycoplasma infection have the ability to induce a scleroderma-like pathology. As mentioned above, autoreactive B lymphocytes generated during infection are normally short lived and destined to undergo apoptosis, but can be employed to generate hybridomas if fused to a bcl-2 expressing plasmacytoma (Ray et al., 1996). The rescue of infection-induced autoreactive B lymphocytes and their transformation into antibody-producing hybridomas may substitute for a pathologic amplification of autoantibody titers, as it occurs during autoimmune disease. Monoclonal anti-centrosome autoantibodies, enriched from hybridoma supernatants may have acquired the ability to transfer disease to healthy recipients.

To directly test the pathogenic potential of autoreactive monoclonal antibodies with anti-centrosome specificities, but also of monoclonal antibodies specific for ER-antigens or possibly for tissue-specific antigens, hybridoma cells or supernatants would need to be injected into healthy recipients. It is possible that the injection of a single monoclonal antibody specificity is not sufficient to induce pathology, but that two or more monoclonal antibodies binding different epitopes of the same autoantigen are required to activate pathogenic immune effector cells, by cross-linking for example Fc-receptors on their surface (Maccioni et al., 2002).

A demonstration of autoantibody-induced scleroderma-like pathogenesis in mice would require a histological evidence of characteristic lesions, occurring subsequent to

autoantibody injection, in the organs affected by the human disease. Specifically, a critical argument for autoantibody-induced pathogenesis would be the immunohistochemical detection of scleroderma-like lympho-monocytic inflammatory infiltrates and, possibly, of injected monoclonal autoantibodies in such lesions. Autoantibody-induced inflammation may subside upon discontinuation of autoantibody inoculations or persist and develop into chronic scleroderma lesions (Matsumoto et al., 2002). Scleroderma-like pathogenesis is expected to occur upon injection of autoreactive monoclonal autoantibodies, but not upon injection of the same amount of an isotype-matched anti-hapten monoclonal antibody control. Such findings can be directly translated to the investigation of a possible role of pathogenic autoantibodies in the development of human scleroderma. Anti-centrosome autoantibodies can be affinity-purified from individual or pooled sera of scleroderma patients and used to immunize mice to generate monoclonal anti-idiotypic antibodies. Anti-idiotypic antibodies would be used for the subsequent immunohistochemical detection of anti-centrosome autoantibodies in human scleroderma lesions.

5. Test for Mycoplasma Infection in Scleroderma. Finally, given that scleroderma patients develop anti-centrosome autoantibodies and that anti-centrosome autoantibody development in mice is due to a mycoplasma infection, a link between mycoplasma infection and human scleroderma may possibly exist. It would be of interest to establish whether scleroderma patients harbor mycoplasma nucleic acid, especially in affected organs. Preliminary results obtained during the course of this study, demonstrating

amplification of mycoplasma sequences from the skin of scleroderma patients but not from unaffected individuals, encourage this approach (Fig. 14).

Fig 14. Search for a human *Mycoplasma* in scleroderma. PCR primers were designed based on pig (P) and mouse (M) *Mycoplasma hyorhinis* sequences of the transcription elongation factor (tuf) gene (a), triosephosphate isomerase (tpi) gene (b) and 16S ribosomal gene (c). Archived human skin was used as a source of DNA in an attempt to amplify *Mycoplasma* tuf, tpi and 16s gene regions. *Mycoplasma hyorhinis* sequences were amplified from 5 of 6 scleroderma patient samples (H, human mycoplasma sequence), but from none of 6 basal cell carcinoma patients and none of 6 patients with allergic skin reactions.

14a.

P-TUF [REDACTED]
M-TUF [REDACTED]
H-TUF [REDACTED]

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF ~~AAATGACGAGGAGTACTGACATTCATGACAGTATGAAATATA~~
M-TUF ~~AATGACGAGGAGTACTGACATTCATGACAGTATGAAATATA~~
H-TUF

P-TUF [REDACTED]
M-TUF [REDACTED]
H-TUF [REDACTED]

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF [REDACTED]
M-TUF [REDACTED]
H-TUF [REDACTED]

P.TUF [REDACTED]
M.TUF [REDACTED]
H.TUF [REDACTED]

P-TUF [REDACTED]
M-TUF [REDACTED]
H-TUF [REDACTED]

P-TUF
M-TUF
W-TUF

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF
M-TUF
H-TUF

P-TUF 
M-TUF
H-TUF 

14b.

P-TPI ATTTGGATTTGCAATTGCAGTCTCCACCATTAAMIGTAGTCGATTAC
M-TPI AATTGATTTTCATGTGCTCTCCTCAATTAATGTTTGCGTTAC
H-TPI

P-TP1 A A A T T A C A A C A A A T T G C A T T A G C A G T C A A A T A T G T C A C A A T T T G
M-TP1 A A A T T A C A A C A A A T T A C A A T T G C A G T C A A A T A T G T C A C A A T T T G

P-TPI: A A A G G G C T A T A C T G C C A A A T T C T G C A A A G T A T T C G A T T A A
 U-TPI: U U U C C C G U A U G U C C U U U U G C U U U U G C U U U U G C U U U U G C U U U U

[illegible]

H-TPI

M-TPI
H-TPI

M-TPI: AAGAGCAAGTCAAGTAATTCGATATCACTTAAAATTTCATTAAAG
H-TPI: AAGAGCAAGTCAAGTAATTCGATATCACTTAAAATTTCATTAAAG

P-TPI
M-TPI
H-TPI

P-TPI ~~ATACAGCAATTAATCTGTACAGCAGAGAACACACACAGGTTTGCAGAT~~
M-TPI ~~ATACAGCAATTAATCTGTACAGCAGAGAACACACACAGGTTTGCAGAT~~

P-TPI: TACGAGAACAAACAATAAATTTAACTATCTTATATGGCGGTGGCT
M-TPI: TACGAGAACAAACAATAAATTTAACTATCTTATATGGCGGTGGCT

P-TPI [REDACTED]

14c.

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

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 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

H-16s [REDACTED]
 M-16s [REDACTED]
 P-16s [REDACTED]

D. Summary

This thesis research contributes to our understanding of autoantibody development by defining:

1. the molecular identity of centrosome antigens targeted by autoantibodies in scleroderma patients.
2. the development in scleroderma patients of autoantibodies to multiple antigens of the same macromolecular centrosomal complex, a prevalence pattern suggestive of intermolecular epitope spreading.
3. the prevalence pattern of autoantibodies to centrosome antigens in relation to human connective tissue diseases including scleroderma, SLE, Sjogren syndrome and rheumatoid arthritis.
4. the specificities and isotypes of autoantibodies present in the natural repertoire of unimmunized mice.
5. the infection-induced development of oligoclonal, class-switched autoantibodies in mice.
6. the amplification of infection-induced autoantibody responses in mice to recruit additional, oligoclonal autoantibody specificities of IgM isotype.

The work presented in this dissertation contributes to our understanding of scleroderma by showing:

1. that the detection of antibodies to centrosomes can orient the diagnosis of human scleroderma, especially in the subset of patients that lack other autoantibody specificities characteristic for the disease.
2. that scleroderma-like autoantibody responses can develop in mice as a consequence of infection, thus incriminating infection as a possible cofactor in scleroderma etiology.
3. that loss of tolerance to centrosome antigens can initiate in mice scleroderma-like autoimmune responses, thus providing an insight into scleroderma autoimmune pathogenesis.
4. that autoantibodies to centrosomes are prevented from developing in mice by antibiotic treatment prior and during mycoplasma infection, thus indicating the possible value of a prophylactic antibiotic treatment of infections in scleroderma.

CHAPTER V

MATERIALS AND METHODS

A. Patients and Patient Sera

Sera from 129 patients with scleroderma-spectrum disorders who received medical care at the University of Connecticut Health Center, or at the Hôpital Notre-Dame in Montréal were examined. Of these, 104 patients fulfilled the American College of Rheumatology (ACR) criteria for scleroderma, 88 patients having proximal scleroderma and 16 patients having limited disease (1980). Twenty-one patients had CREST syndrome (two or more features: calcinosis, Raynaud's phenomenon, esophageal dysmotility, sclerodactily, telangiectasias who do not fulfill criteria for SSc). Three patients had overlap syndrome: one had proximal scleroderma and systemic lupus erythematosus, one had proximal scleroderma and sicca syndrome, and one had CREST and sicca. One patient had linear scleroderma. Sera from these patients were stored at -70°C until use. The SSc sera were tested previously for other autoantibodies including anti-nuclear antibodies (ANA), anti-topoisomerase I, anti-centromere (ACA), anti-double-stranded DNA, anti-small nuclear ribonucleoproteins, and anti-Sm, anti-La, and anti-Ro autoantibodies (Galperin et al., 1996; Okano, 1996; Vazquez-Abad and Rothfield, 1995; Vazquez-Abad et al., 1994).

Sera from patients with other connective tissue diseases (systemic lupus erythematosus, rheumatoid arthritis, dermatomyositis, polymyositis, and Sjogren's syndrome) were obtained from the University of Connecticut Health Center and from Dr. T. Medsger

(University of Pittsburgh). Normal sera were collected from blood donors at the University of Connecticut Health Center and at the University of Massachusetts Medical Center.

B. Mice and Mouse Sera

Balb.c/SvJ, C57BL/6J and 129/J mice were purchased from the Jackson Laboratories (Bar Harbor, ME), Charles River Laboratories (Wilmington, MA) and Taconic Farms (Germantown, NY). Uninfected mice were housed in a pathogen free facility, while infected mice were housed under biocontainment conditions at the University of Massachusetts Medical Center. Sera were also obtained from mouse colonies housed under conventional conditions at the University of Massachusetts Medical Center and the University of Connecticut Health Center.

C. Cell Lines and Tissue Culture

HepG2 is a human hepatoma cell line; 293T is a human epithelial kidney cell line; NIH3T3 is a murine fibroblast cell line; SP2 is a murine plasmacytoma; Vero is a fibroblast cell line derived from African green monkey kidney. Cells were maintained in a 5%CO₂ incubator at 37°C in Dulbecco's minimal essential media (DMEM, Gibco, Grand Island, NY) supplemented with 100 U/ml of penicillin G (Gibco, Grand Island, NY), 100 µg/ml streptomycin sulfate (Gibco, Grand Island, NY), 2 mM L-glutamine (Gibco, Grand Island, NY) and 10% heat inactivated (56°C, 30 min), certified

Mycoplasma-free fetal bovine serum (Gibco, Grand Island, NY). All tissue culture reagents, trypsin (Gibco, Grand Island, NY) included, were tested for Mycoplasma contamination using a commercially available PCR kit (Stratagene, La Jolla, CA).

D. Preparation of Recombinant Protein

We used cDNAs isolated in the original screen with SSc autoimmune serum that encoded truncated forms of the centrosome proteins pericentrin (73 kD, clone λ 1.1) and centriolin (CP140, 75 kD) (Doxsey et al., 1994). Recombinant proteins were insoluble and were purified as inclusion bodies as described (Doxsey et al., 1994). Antibodies against the bacterial fusion proteins were raised as described (Doxsey et al., 1994). Recombinant proteins served as autoantigens in the biochemical assays and antibodies were used in double-label IF experiments.

E. Antibodies

Mouse monoclonal antibodies to *Mycoplasma hyorhinis* variable lipoprotein were a gift of Dr. K. S. Wise of the University of Missouri. Rabbit polyclonal antibodies to *Mycoplasma hyorhinis* were purchased from Cortex Biochem (San Leandro, CA). A rabbit anti-pericentrin antibody was used as a centrosome marker. A rabbit anti-calnexin antibody (Stressgen, Victoria, BC Canada) and a mouse monoclonal IgG2a anti-BiP

antibody (BD Biosciences, Franklin Lakes, NJ) were used as a markers for the endoplasmic reticulum.

F. Immunoblotting

Fifty micrograms of recombinant fusion protein was heated to 90°C in 200 µl of sample buffer for 3 min, subjected to electrophoresis on a 12% sodium dodecyl sulfate (SDS)-polyacrylamide curtain gel (without wells), and transferred to Immobilon membrane (Millipore Corporation, Bedford, MA) at 150 mA for 1 h in a semidry blotter (Fisher Biotech, Pittsburgh, PA) at room temperature (RT). The membrane was incubated in Tris buffered saline (TBS) pH 7.4 containing 1% bovine serum albumin (BSA, Sigma, St. Louis, MO) at RT for 60 min or at 4°C overnight. The membrane was then washed in TBS with 0.05% Tween (TBST) for 20 min and subsequently incubated with patient sera diluted to 1/250 in TBST-BSA at RT for 60 min. Assays of autoantibodies in mouse sera employed serially diluted samples starting at a dilution of 1/40 in TBST-BSA at RT for 60 min. A multiwell miniblotted (Integrated Separation Systems, MA) was used to probe 56 sera at one time. The incubation step was followed by washing in TBST for 60 minutes with three changes. The membrane was incubated for 60 minutes at room temperature with horse radish peroxidase (HRP)-coupled anti-human secondary antibodies (Sigma, St. Louis, MO) or anti-mouse secondary antibodies (Amersham, Piscataway, NJ) and washed in TBS. Bound secondary antibodies were detected by chemiluminescence according to the manufacturers protocol (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

G. Immunofluorescence

Human sera were assayed on cultured human cell substrates. HepG2 cells grown on coverslips were washed twice in phosphate-buffered saline (PBS) pH 7.35 and permeabilized in Pipes (80 mM), EGTA (5mM), $MgCl_2$ (1mM), Triton X-100 (0.5%) for 45 seconds at RT and fixed in methanol at $-20^{\circ}C$ for 5-10 min. Fixed cells were washed once in PBS and twice in PBS with 1% BSA and 0.5% Triton X-100 (PBSAT) and subsequently incubated with patient sera at a dilution of 1:250 in a humidified chamber for 40 minutes at RT.

Mouse sera were assayed on cultured mouse NIH3T3 or monkey Vero cell substrates. NIH3T3 cells or Vero cells were grown on coverslips, washed in PBS, pH 7.35 and fixed in methanol at $-20^{\circ}C$ for 10 min or in 4% paraformaldehyde (Electron Microscopy Science, Ft. Washington, PA) in PBS at RT for 30 minutes. Fixed cells were washed in PBS containing 0.1% BSA and 0.25% Triton X-100 (PBSAT) and subsequently incubated with freshly prepared serially diluted mouse sera in a humidified chamber for 40 min at RT.

Marker antibodies were incubated for 20 minutes at RT.

Cells were washed 5-10 times with PBSAT and subsequently incubated for 20 min with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG and Cy3-conjugated donkey anti-human secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) or anti-mouse secondary antibodies (see below) at dilutions of 1:500 in PBSAT. Cy3-coupled donkey anti-mouse antibodies specific for IgG (H+L) or IgM were

purchased from Jackson ImmunoResearch Laboratories (West Grove, PA), as were Cy3- and biotin-coupled goat anti-mouse antibodies specific for Fc γ used to detect autoantibodies of IgG isotype, or Cy3-coupled anti-goat antibody used for signal amplification in some experiments. Rat anti-mouse IgG1, IgG2a and IgG2b purchased from Pharmingen (San Diego, CA), rabbit and rat anti-mouse IgG1 and IgG2b purchased from Zymed (San Francisco, CA) were used to define autoantibody subclasses. Secondary antibodies were washed 5-10 times with PBSAT and once with PBS. Nuclear DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, 5pg/ml in PBS) for 1 min. Coverslips were washed once with PBS, briefly with water, mounted using Vecta Shield (Vector, Burlingame, CA) or ProLong Antifade kit (Molecular Probes, Eugene, OR) and viewed on an Axiophot microscope (Zeiss, Germany).

Human samples were scored open-label and positive samples were confirmed by a second investigator. Comparisons between infected and uninfected mouse groups were scored open-label. Results were confirmed blindly by one or two investigators.

H. Virus and Mycoplasma Stocks

Herpes simplex virus I (KOS 1.1 strain) and Sindbis virus stocks have been provided by Dr. R. Welsh (University of Massachusetts Medical Center). To prepare stocks of murine mycoplasma, Vero cell supernatants are harvested 7 days after inoculation with 10^3 pfu of mycoplasma/T150 flask. After a preliminary centrifugation in closed 50 ml tubes at 2000 rpm, 4 °C for 20 min, infected supernatants are loaded gently on 1.5 ml cushions of 70% renografin in PBS, added at the bottom of centrifuge tubes (25 x 89 mm, Beckman

Instruments, Inc., Palo Alto, CA) and centrifuged at 28,000 rpm in an SW28 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 4 °C for 90 min. The fraction harvested at the renografin-media interface is subsequently added to the top of a 10-70% continuous renografin gradient and centrifuged at 28,000 rpm in an SW28 rotor at 4 °C for 12 h. The refractive index of gradient fractions is determined using a refractometer. Murine mycoplasma stocks in sucrose, renografin or DMEM were maintained at -80°C. Lyophilized pig *Mycoplasma hyorhinis* was purchased from ATCC.

I. Mouse Infection

Six weeks old male or female mice were prebled and anesthetized each with 1 mg Nembutal (Abbott Laboratories, Chicago, IL). Mice were infected intranasally with 10^4 - 10^9 pfu/ml of 1x or 4x plaque-purified inoculum of infectious agent that had been partially purified on a renografin gradient (Squibb Diagnostics, New Brunswick, NJ) or sedimented on a cushion of 70% sucrose in PBS. Centrosome autoantibody production was observed at mycoplasma titers between 10^4 - 10^9 plaque-forming units (p.f.u.). The efficiency of autoantibody production in experimental groups (50-75% of the animals) did not increase at higher titers and was not observed below 10^4 p.f.u. Similar results were observed in many mouse strains including Balb.c/SvJ, C57BL/6J and 129/J mice. Control mice were inoculated intranasally with uninfected culture supernatant that had been either spun on a renografin gradient or harvested at the sucrose interface. Mouse-to-mouse contamination was avoided by using individual, disposable equipment, including

restrainers. Inoculated mice were subsequently bled every 2 or 3 weeks and sera were assayed for autoantibody development. Ciprofloxacin (20 $\mu\text{g/g}$ body weight) was delivered orally by gavage once per day.

J. Plaque Assay

To obtain organ homogenates, mouse spleens were placed in 1 ml of cold media and homogenized in a mortar and pestle device. The spleen homogenates were centrifuged at $1075 \times g$ (2000 rpm) at 4°C for 20 min to obtain clarified supernatants.

Freshly confluent cell monolayers were grown on 6-well plates. Ten-fold serial dilutions of infectious agent inoculi were prepared. Diluted inoculi contained in 0.1 ml were added to 1 ml fresh media per well and cells were incubated for 90 min in a humidified 5% CO_2 incubator at 37°C . Monolayers were overlaid with 4 ml/well EMEM media (Eagle's modified essential media, BioWhittaker, Walkersville, MD) containing 0.5% agarose (SeaKem, BMA Rockland, ME) and supplemented with 5% certified Mycoplasma-free fetal calf serum (Gibco, Grand Island, NY), 100 U/ml of penicillin G (Gibco, Grand Island, NY), 100 $\mu\text{g/ml}$ streptomycin sulfate (Gibco, Grand Island, NY), 2 mM L-glutamine (Gibco, Grand Island, NY), and 2.5 U/ml nystatin (Gibco, Grand Island, NY). Monolayers were incubated at 37°C and six days later 2 ml of 0.5% agarose in EMEM containing 0.3 mg Neutral Red (Sigma, St. Louis, MO) were added to each well. For mycoplasma titration, plaques were counted seven days post-inoculation. For Sindbis

virus titration, plaques were counted 2 days post-inoculation. For herpes simplex virus I titration, plaques were counted 3-4 days post-inoculation.

The murine mycoplasma grows in NIH3T3, Vero, 293T and SP2 supernatants and plaques on NIH3T3, Vero and 293T monolayers. Ciprofloxacin (ICN Biomedicals, Irvine, CA) was used at a concentration of 0.5 µg/ml. Doxycycline (Sigma, St. Louis, MO) was used at a concentration of 1 µg/ml. Antibiotic was added to mycoplasma infected cultures for 24 hours or 7 days and supernatants were subsequently titrated by plaque assay.

K. Cloning, PCR, Sequencing and Sequence Alignments

DNA was extracted from gradient purified murine mycoplasma and from lyophilized *Mycoplasma hyorhinis* supplied by ATCC (Rockville, MD) by using a proteinase K digestion protocol (Qiagen Inc., Valencia, CA). Genomic mycoplasma DNA was cleaved with EcoRI or HindIII (N.E.B., Beverly, MA), the digest was visualized on 1% agarose/TAE gels, purified and randomly cloned into pUC18 vector. Plasmid DNA from individual clones was purified using QIAprep columns (Qiagen Inc., Valencia, CA) and the nucleotide sequences were determined using adequate vector primers at the DNA Sequencing Facility of the University of Massachusetts Medical Center. NCBI databases were searched for homologous sequences using BLASTX (<http://www.ncbi.nlm.nih.gov/blast>). Comparing each mycoplasma sequence to public databases resulted in a list of alignments. Primers were designed based on these

alignments and the corresponding genes were specifically amplified from mouse and pig *Mycoplasma hyorhinis* strains and compared. Primer sequences are as follows: for the 16s gene 5'-GGTTAAGTCCTGCAACGAGC-3' and 5'-GTTAACCTCACC GACTTTGGG-3'; for the tuf gene 5'-GGCTTGGTGCTGCTCAAATGGA-3' and 5'-CCTACAGTTCTACCACCTTCACGG-3'; for the putative methylase gene 5'-GATAATACAAGAAGTGGTTTATTGC -3' and 5'-AAAACCTTTCCAACTCGAGTT- ATATCC-3'; for the putative dehydrogenase gene 5'-TGAAGAACTTTAGATGTTTCAACAACTCC-3' and 5'-TCCTGTTGATTTTTCTACATTC-3'; for the putative permease gene 5'-CCAGTTTTTGTAGATATTAAAGAAATCG-3' and 5'-CTGTAGCTGCAAAAAATCC-3'; for the tpi gene 5'-ATTTGGATTTTGCAATTGC-3' and 5'-TTTTCTTGCGAAACTGAGCCAACC -3'.

Each PCR reaction was prepared in a laminar flow PCR hood (AirClean, Raleigh, NC) and used 50 pmol of each primer, 5U of HotStarTaq polymerase (Qiagen Inc., Valencia, CA), 100 mM of each deoxynucleotide triphosphate, in 67 mM Tris buffer (pH8.8), 4 mM MgCl₂, 16 mM (NH₄)₂SO₄, 10mM 2-mercaptoethanol and 100 (g/ml bovine serum albumin, BSA).

PCR amplifications were performed at 95 °C for 5 min (1 cycle), 94 °C for 1 min, 50 °C for 2 min, 72 °C for 1 min (40 cycles) and 72 °C for 5 min (1 cycle). Amplifications were carried out in an MJ Thermocycler (MJ Research, Watertown, MA). Gel purified PCR amplification products were cloned using a TA cloning system (Invitrogen, Carlsbad,

CA) according to the manufacturer's instructions. PCR detections of mycoplasma in mouse tissue were performed by two independent investigators.

L. Electron microscopy

After plaque-formation, monolayers were fixed and embedded in situ. Mycoplasma was pelleted from enriched gradient fractions, fixed and embedded. Fixation was performed in 4% paraformaldehyde (Electron Microscopy Science, Ft. Washington, PA) in PBS at RT for 30 minutes. Samples were processed for electron microscopy as described (Doxsey et al., 1994). Thin sections (500-700 nm) of the embedded samples were cut on a Sorvall ultramicrotome, stained with uranyl acetate and lead citrate (Ted Pella Incorporated, Redding, CA) and viewed in a JOEL electron microscope (Doxsey et al., 1994).

M. Statistical Analysis

Analysis of human patient groups. The Z test was used to determine whether statistical differences existed between (a) the proportion of SSc patients and normal individuals autoreactive to centrosome proteins, (b) the proportion of SSc patients and patients with other connective tissue diseases autoreactive to centrosome proteins, and (c) the proportion of normal individuals with ANA and those autoreactive to centrosome

proteins. The chi-square test was used as an independence test to compare the results obtained by IF with the results obtained by immunoblotting for the same groups of SSc patients listed above. The chi-square test was also used to compare the numbers of patients positive for centrosome autoantibodies that had different forms of the disease or different clinical disease features. Sensitivity and specificity of diagnostic tests were calculated using our results and published data according to published definitions and formulas (Knapp and Miller, 1992; Vazquez-Abad and Rothfield, 1995).

Statistical analysis mouse serum autoantibody titers was performed using an Epi Info 6.1 software package. Comparisons between autoantibody titers in infected and uninfected mice were performed using the Mann-Whitney test. The Student t-test, two-tailed with unequal variances was used where appropriate.

CHAPTER VI

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